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13. ABSTRACT *(Maximum 200 Words)*

Recently we identified a novel vitamin D analog, 1a-hydroxy-24 ethyl vitamin D_5 ($1\alpha(OH)D_5$) that showed potent growth inhibitory and cell-differentiating actions in breast cancer cells. Based on our findings in in vitro and in vivo experimental model systems, we hypothesized that $1\alpha(OH)D_5$, when administered to women with breast cancer, will induce differentiation of dedifferentiated cells and thereby prevent progression of malignancy. The major tasks proposed for 1999-2000 are 1) perform preclinical toxicity study of $1\alpha(OH)D_5$ according to GLP; 2) determine in vitro effect of $1\alpha(OH)D_5$ on normal and malignant breast tissues; 3) prepare $1\alpha(OH)D_5$ according to GMP for future phase I clinical trial. We completed the preclinical study in rats. Our results show that $1\alpha(OH)D_5$ has no serious toxicity; a hypercalcemic effect was observed at high dose, which was reversible. In vitro study in tissues obtained from patients show that $1\alpha(OH)D_5$ has no effect on normal breast epithelial cells, but it induces apoptosis in breast cancer. It also showed apoptotic effect in fibroadenomas. We have completed 5 steps in the synthesis of $1\alpha(OH)D_5$ for preparation of $1\alpha(OH)D_5$ for phase I clinical study.

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Introduction

Although most patients with localized breast cancer can be adequately treated with some combination of surgery and radiation therapy, a vast majority of patients with local, loco-regional, or metastatic breast cancer need additional use of chemotherapeutic agents and/or hormonal therapy. While initially responsive to various cytotoxic and hormonal modalities, most breast cancers ultimately acquire resistance to current systemic therapies. Thus, development of novel effective therapeutic and preventive agents is critical. Recently, several vitamins and vitamin analogs have been foci of investigation as therapeutic and chemopreventive agents for a number of malignancies. Amongst various agents, analogs of vitamin A and D have shown the most promising results.[1,2] The active metabolite of vitamin D_3, 1α25 dihydroxy D_3 ($1\alpha,25(OH)_2D_3$), has been conclusively shown to induce differentiation *in vitro* in a variety of cancers, including breast cancer.[3,4,5] However, many of these compounds highly efficacious in experimental systems did not progress from bench to the bedside, due to their hypercalcemic activity. Numerous analogs of vitamin D_3 have been synthesized in search of a non-calcemic or relatively less calcemic vitamin D with similar antiproliferative effects.[6,7,8,9,10,11,12,13,14] Recently, we synthesized a novel analog of 24-ethyl-vitamin D_3 (vitamin D_5, Fig. 1), 1α hydroxy-24-ethyl-vitamin D_3 ($1\alpha(OH)\ D_5$), and evaluated its chemopreventive and chemotherapeutic actions in experimental models.[15,16] For the chemopreventive effect, we used the mammary gland organ culture model established by Dr. R.G. Mehta (Co-Investigator). To determine the effect on breast cancer cells, we used ER+VDR+ (MCF-7, ZR-&5-1), VDR+ER- (UISO-BCA-4), and ER-VDR- (MDA-MB-231) cell lines.

Previous results from our laboratory with $1\alpha(OH)D_5$ are summarized below.

- $1\alpha(OH)D_5$ has chemopreventive action in mouse organ culture model (15).
- $1\alpha(OH)D_5$ has both growth inhibitory and cell differentiating actions in human breast carcinoma cell lines (16,17).
- $1\alpha(OH)D_5$ administered s.c. or supplemented in the diet inhibits the in vivo growth of human breast carcinoma transplanted into athymic mice (16).
- Preliminary studies in small groups of animals (mice and rat) suggested that $1\alpha(OH)D_5$ is not toxic in mice and rats.
- $1\alpha(OH)D_5$ is metabolized into two major metabolites (1,24 or 1,25 vitamin D5) in human breast tumors/nonmalignant breast tissues.

$1\alpha(OH)D_5$ was proven to have potent chemopreventive action against mammary carcinogenesis in experimental animals; it has only antiproliferative action in ER+ breast carcinoma cells and cell-differentiating action in both ER+ and ER- breast cancer cells. In vitro studies indicated that several biomarkers, such as enhanced expression of ICAM-1, nm23, α2 integrin, and increased accumulation of casein and lipids, are associated with positive response to $1\alpha(OH)D_5$. Among these biomarkers examined (all cell-differentiating), increased α2 integrin and casein levels were found to be the most reliable and sensitive parameters indicating response of this drug. The compound was well tolerated without any serious toxicity in rats and mice.

In the proposed study, we aimed to evaluate the chemotherapeutic properties of $1\alpha(OH)D_5$. Using breast tumors with different histologic subtypes and different molecular and biological characteristics, we aim to evaluate the effects of $1\alpha(OH)D_5$ on the growth, differentiation, and progression of breast cancer in vitro in explant culture and in vivo in the athymic mouse model. We aim to confirm α2/casein enhancement as predictive markers for response to $1\alpha(OH)D_5$ in breast cancer patients. If the compound is found safe and effective in experimental systems, we aim to initiate a phase I/II clinical trial to determine the dose tolerance and efficacy of $1\alpha(OH)D_5$ in breast cancer patients. Based on the results of this phase I/II trial, our long-term objective is to initiate a multi-center chemoprevention trial in high-risk individuals.

Hypothesis proposed

We hypothesize that (1) $1\alpha(OH)D_5$ administered to women with breast cancer will induce differentiation of dedifferentiated malignant cells and thereby prevent progression of malignancy, and (2) in women with premalignant lesions, $1\alpha(OH)D_5$ will prevent dedifferentiation and thus prevent induction and/or development of breast cancer.

Technical Objectives proposed

The specific objectives of the proposed study are to:

1. Establish and evaluate biomarkers predicting $1\alpha(OH)D_5$ response in malignant breast cancer and DCIS (Ductal Carcinoma in Situ).
2. Study the molecular mechanism by which $1\alpha(OH)D_5$ induces differentiation/inhibits proliferation of breast cancer cells.
3. Perform (according to FDA requirement) preclinical toxicity and pharmacokinetic studies of $1\alpha(OH)D_5$.
4. Initiate a phase I/II trial in advanced breast cancer patients. (During this trial, we will also obtain data on the metabolism of $1\alpha(OH)D_5$ in humans).

Successful completion of the proposed study will identify a new chemotherapeutic and possibly chemo-preventive agent in breast cancer.

Statement of work and time schedule proposed for 1999-2000

0-6 months: Accrue breast tumors, normal breast tissues, and DCIS for in vivo and in vitro/in vivo study described in Specific Aim 1. Incubate the tumor/normal breast/DCIS with/without $1\alpha(OH)D_5$; evaluate morphological and biochemical changes in markers of cell proliferation and cell differentiation. Also, initiate in vivo xenograft study, to determine the effect of $1\alpha(OH)D_5$ supplementation on tumor growth and biological parameters. Begin toxicity study mentioned in Specific Aim 3. Study mechanism of $1\alpha(OH)D_5$ action.

7-12 months: Obtain sufficient amount of $1\alpha(OH)D_5$ synthesized under GMP, to initiate toxicity studies. Toxicity experiments described in Specific Aim 3 will be initiated. Metabolism experiments will be conducted in vitro, and identification of metabolites by mass spectrometry will be carried out. Establish cell cycle parameters in response to $1\alpha(OH)D_5$ in ER+ and ER- cell lines.

Results

Task-1: Accrue breast tumors, normal breast tissues, and DCIS for in vivo and in vitro/in vivo study described in Specific Aim 1. Incubate the tumor/normal breast/DCIS with/without $1\alpha(OH)D_5$; evaluate morphological and biochemical changes in markers of cell proliferation and cell differentiation. Also, initiate in vivo xenograft study, to determine the effect of $1\alpha(OH)D_5$ supplementation on tumor growth and biological parameters. Begin toxicity study mentioned in Specific Aim 3. Study mechanism of $1\alpha(OH)D_5$ action. Specific Aim 1 is designed to answer questions related to potential clinical translational applications of $1\alpha(OH)D_5$ in prevention and therapy of breast cancer.:

Due to an unanticipated hold during 1999-2000 on all IRB protocols at UIC, we were able to obtain only a limited number of tumors and normal tissues. For this specific aim, we accrued several malignant breast tissues (n= 13), nonmalignant breast tissues (n=24), and fibroadenomas (n=2) from women with confirmed diagnosis of the disease. Paired malignant and nonmalignant breast tissues (n=10) were obtained from women undergoing mastectomy for confirmed diagnosis of breast cancer. The effects of $1\alpha(OH)D_5$ were determined *in vitro* in explant culture of these tissues.

For *in vitro* experiments, fresh tissues received from the OR were minced into small pieces (~2-3 mm in size). A small piece was fixed immediately in 10% buffered formalin for histopathological evaluation, and the remaining tissues was processed for culture experiments and/or *in vivo* experiments. We confirmed our previous findings for experimental conditions. All tissues were incubated in an atmosphere of 95% O_2 and 5% CO_2. We incubated the tumor/tissue explants in basal culture medium at 37°C alone, or in the medium

containing varying concentrations of $1\alpha(OH)D_5$ (0.1-1 μM) or $1\alpha,25(OH)_2D_3$(0.1 μM) for 0-72 hours. At the end of incubation, the tissues were fixed in 10% buffered formalin and processed for histopathological or immunohistological examination of various biomarkers (Ki-67, VDR, casein, and alpha2 integrin expression).

Effect of $1\alpha(OH)D_5$ on nonmalignant breast tissue

Our results in the current studies clearly show that human breast tissues, both malignant and nonmalignant, preserve the original histological features when incubated in culture medium containing dextran-coated charcoal stripped serum. Histopathology of the normal breast tissue showed that alveolar and ductal morphology is preserved up to 72 hours. When normal breast tissues were incubated in $1,25(OH)2D_3$ or $1\alpha(OH)D_5$, no histological changes were observed in ductal and alveolar structures. Plate 1 shows representative nonmalignant breast tissue following incubation (0-72 hrs) in the culture medium alone or in the medium containing $1,25(OH)2D_3$, 0.1 μM $1\alpha(OH)D_5$, or 1 μM $1\alpha(OH)D_5$. In normal tissue, no significant effect of $1\alpha(OH)D_5$ or vitamin D3 was observed. In all the tissues, viable breast epithelial structures are observed. Occasionally, the presence of cells with pyknotic nuclei was observed in both control and treated cells.

To further confirm the viability of the cells, we examined the immunoreactivity to Ki-67 antibody, a marker for cell proliferation. Originally, in breast tissue obtained from women (0 time), we observed 4-6 cells/each alveolar section undergoing proliferation, as evident from the immunoreactivity to Ki-67. This proliferation rate was maintained throughout the course of study in all control and treated tissues (Plate 2).

We also examined expression of intracellular expression of casein in nonmalignant tissues incubated in vitro in the presence/absence of vitamin D analog. In original tissue (0 time), no detectable expression of casein was observed immunohistochemically. Similarly, in tissues incubated for 24-72 hours in the control medium, no detectable casein immunoreactivity was observed. Enhanced immunoreactivity to casein antibody was observed in tissues incubated with $1\alpha(OH)D_5$ for 72 hours (Plate 3).

Changes in the expression of Vitamin D receptor (VDR) are shown in nonmalignant breast tissues in Plate 4. All alveolar and ductal lesions showed distinct immunostaining for VDR localized in the nucleus. No change in VDR expression was observed in control or treated tissues during the course of study.

In summary, our results in nonmalignant breast tissues show that nonmalignant breast tissues cultured in vitro maintained the alveolar and structural integrity up to 72 hours; cells are viable and proliferating. VDR is present in both alveolar and ductal cells, and it is maintained in the current culture conditions used. $1\alpha(OH)D_5$ had no effect on the cellular morphology or proliferation on breast epithelial cells, but it probably increases the expression of casein; however, these results need to be confirmed in a large number of specimens.

Effect of $1\alpha(OH)D_5$ on fibroadenoma

Fibroadenomas maintained in culture not only showed viability of the individual cells, but also preserved the essential architecture of the alveolar, ductal, and stromal components. Only occasionally, cells containing pyknotic nuclei were seen. However, when $1\alpha(OH)D_5$ was added to the medium 72 hours later, a proportion of the alveoli lost their epithelial components. The individual cells showed karyolysis and nuclear pyknosis with patchy areas of degeneration in the stromal components (Plate 5, Figs A and B). Similar to the normal breast tissue, Ki67 immunoreactivity did not show any changes after incubation with $1\alpha(OH)D_5$ (Plate 5 C & D). In contrast, VDR expression appears to be enhanced by at least 60% in fibroadenomas grown in the medium containing $1\alpha(OH)D_5$ (Plate 5 Figs E &F).

We examined the effect of $1\alpha(OH)D_5$ on fibroadenoma of the breast obtained from women with confirmed diagnosis of the disease. Fibroadenoma tissues were viable when maintained in culture for 72 hours (Plate 5A, B). At 72 hours in tissues incubated in control medium, we observed the presence of normal alveolar and ductal structures, and stromal elements. Breast alveolar structures consisted of distinct basement membrane; epithelial cells surrounded the lumen. All alveolar and ductal cells showed distinct nucleus and abundant cytoplasm. Occasionally, the presence of cells with pyknotic nucleus was observed. In tissues treated with $1\alpha(OH)D_5$ in vitro for 72 hours, many of the alveolar structures appeared to be normal; however, some lost

their epithelial components. Many epithelial cells showed nuclear pyknosis or karyolysis. In the areas with degenerated alveolar structures, stromal components also showed necrotic changes (Plate 5B).

We further examined Ki-67 immunoreactivity in control and 1α(OH)D_5-treated tissues. In control tissue, numerous alveolar epithelial cells and stromal cells showed Ki-67 immunoreactivity. In 1α(OH)D_5-treated tissue, immunoreactivity to Ki-67 was similar to that of control in areas showing no degenerative changes (Plate 5C, D).

Plates 5E and 5F show VDR expression in alveolar structures in control and treated fibroadenoma. In treated tissue, only those areas that showed histopathology similar to that of control tissues showed distinct nuclear localization of VDR. In general, in control tissue, 30-40% of breast epithelial cells showed VDR expression, whereas in treated tissue VDR expression was observed in 90-95% of cells (Plate 5F).

Effect of 1α(OH)D_5 on human breast carcinoma.

We examined the effect of 1α(OH)D_5 in human breast carcinoma tissues incubated in vitro in control and 1 μM 1α(OH)D_5-containing medium. In most of the malignant breast tumors studied, the original histopathological features were preserved up to 48 hours when tissues were incubated in the control medium. Plate 6 shows the representative histopathology of a tumor incubated in vitro in control medium for 48 hours. Very few cells in this control tissue showed apoptotic or pyknotic changes (Plate 6A). In contrast, cells incubated for 48 hours in medium containing 1 μM 1α(OH)D_5 contained apoptotic cells (Plate 6B). In addition, many cells at various stages of apoptotic death were observed. We are currently studying Ki-67, VDR, and casein expression in tumor tissues.

In previous studies, we showed that, among the various breast cell differentiation-associated biomarkers studied, increased α2 integrin and casein levels were found to be the most reliable and sensitive parameters indicating response to 1α(OH)D_5. We studied alpha2 integrin expression in paraffin sections of human breast carcinomas and nonmalignant breast tissues. Although the alpha2 antibody used in our studies is highly recommended for immunohistochemistry, we were unable to observe specific staining for integrin in any tissues studied. We tested several antigen retrieval systems (citrate buffer, protease digestion, trypsin digestion, SDS treatment, microwave techniques) with non-reliable results. Currently, we are analyzing alpha2 integrin expression in frozen tumor/nonmalignant breast tissues.

Studies of other prognostic markers in primary breast carcinomas

All primary breast carcinomas are analyzed for the expression of various prognostic markers (ER, PR, and p53 protein).

***In vivo* study:**

We had sufficient tumor tissue available after performing in vitro studies from 3 patients. We first developed a primary culture from the breast tumor, and then the cells growing in culture were injected into 2-3 animals. The cell suspension was mixed with Matrigel (1:1 vol.), then injected s.c. near the mammary fat pad into 3- to 4-week-old female athymic mice (at least 4 animals/tissue). Two of the 3 primary cultures developed xenografts. The xenografts were further propagated into 10 athymic mice. Animals were divided into two groups 1) receiving control diet 2) receiving vitamin D-supplemented (25 μg/kg-diet) diet. These experiments are in progress currently.

We have already shown that the dose selected for 1α(OH)D_5 is nontoxic and is effective for inhibiting the growth of various breast cancers transplanted into athymic mice.

Mechanism of 1α(OH)D_5 action: In vitro studies using established breast carcinoma cell lines

Previously we showed that the antiproliferative effect of 1α(OH)D_5 is more prevalent in ER+ cells and that the cell-differentiating effect is more common in VDR+ cells, irrespective of their ER status. We hypothesize that vitamin D_5 or its metabolite induces differentiation in all breast carcinomas positive for VDR, whereas in ER+ tumor, it has an additional antiproliferative effect being mediated by an interaction with estrogen receptor. We attempted, in this funding period, to answer the following questions: Is the 1α(OH)D5-

mediated, antiproliferative effect in ER+ breast cancer cells due to the interaction of 1α(OH)D5 with estrogen receptors? Is the effect of 1α(OH)D5 on the induction of differentiation in both ER+ and ER- cells mediated through its interaction with VDR?

In order to achieve this aim, we used the commercially available human breast carcinoma cell line MDA-MB-231. MDA-MB-231 cells show low/undetectable levels of VDR. This cell line shows no growth inhibitory effect to $1\alpha(OH)D_5$ (Figures 1 and 2).

During this funding period, we transfected MDA-MB-231 cells with various cDNA constructs: 1) Vector with ampicillin resistance gene only; 2) vector with human VDR cDNA construct; 3) Vector with VDRE c DNA construct; and 4) Vector with both VDR and VDRE inserts. The cDNA transfection was carried out using the Lipofectin method. Cells were subjected to G-418 resistance, and colonies resistant to G-418 were selected and expanded into cell lines. We examined the expression of VDR in all cell lines by immunohistochemistry and by western blot analysis. We are unable to observe enhanced expression of VDR in any of the transfectants. Currently, we are analyzing m-RNA for VDR to determine whether transfection of these genes was achieved. It is also possible that expression of oncogenes such as H-ras in MDA-MB-231 is interfering with VDR expression in these cells.

Is $1\alpha(OH)D_5$ action mediated through ER?

We examined the effect of $1\alpha(OH)D_5$ in parental MDA-MB-231 cells and in those transfected with cDNA for ER (ER alpha). The cell line showing nuclear expression of ER (S-30) was kindly provided to us by Dr. Craig Jordan, Northwestern Medical School, after obtaining written permission from Dr. J. Chambon. These series of in vitro studies were performed to determine whether the effect of $1\alpha(OH)D_5$ is at least partially mediated through estrogen receptors.

Effect of $1\alpha(OH)D_5$ on in vitro growth of MDA-MB-231 and S-30 (MDA-MB-231 with ER) cells

For in vitro growth, cells (20,000) were plated in 24-well culture plates and then exposed to control medium (with stripped serum only) or medium containing various molar concentration of $1\alpha(OH)D_5$ or $1,25(OH)2D_3$. We also included estradiol, tamoxifen, and a combination of all these additives (shown in Figures 2 and 3) as treatment groups. Medium was changed on day 4 and 7 after initiating treatment, and the number of cells was counted on day 10 using a coulter counter. Data represent mean cell number of quadruplet observations. As shown in Figure 2, the growth of MDA-MB-231 was not affected significantly by $1\alpha(OH)D_5$ or $1,25(OH)2D_2$ treatments. We also failed to show a significant effect of estradiol, tamoxifen, or a combination of the latter components with $1\alpha(OH)D_5$. The effect of various combination treatments on S-30 cells is shown in Figure 3. In S-30 cells, tamoxifen was effective in inhibiting the estradiol-induced cell growth. 1,25 D3 or $1\alpha(OH)D_5$ alone had no effect on the cell growth. Interestingly, combination treatment with estradiol, tamoxifen, and 1(OH)D5 significantly reduced cell growth compared to that of control or estradiol alone or estradiol+tamoxifen treatment. Our results in this study suggest that $1\alpha(OH)D_5$ does not have ER-mediated action on S-30 cells. Alternatively, the S-30 cell line with exogenously transfected ER cDNA fails to respond to $1\alpha(OH)D_5$ as native ER positive cells would. It is also possible that S-30 cells are unable to metabolize $1\alpha(OH)D_5$. Dr. Reddy is currently studying $1\alpha(OH)D_5$ metabolism in ER-positive and ER-negative breast carcinoma cell lines, including MDA-MB-231 and S-30.

In addition to growth inhibitory action, we evaluated the effect of $1\alpha(OH)D_5$ on expression of alpha2 integrin in S-30 and MDA-MB-231 cells. Alpha2 integrin was measured by flow cytometry. In general, S-30 cells had significantly higher alpha2 integrin levels than their parental MDA-MB-231 cells (Figure 4a). The promoter region of alpha2 integrin is reported to contain an ER-binding site (18); thus, the presence of ER even in the absence of its ligand could enhance the expression of alpha2 integrin. $1\alpha(OH)D_5$ treatment increased alpha2 integrin levels more so than in control untreated cells. No such effect was observed in MDA-MB-231 cells (Figure 4b).

Task-2 7-12 months: Obtain sufficient amount of 1α(OH)D_5 synthesized under GMP, to initiate toxicity studies. Toxicity experiments described in Specific Aim 3 will be initiated.

Preclinical Toxicity under GLP:

The preclinical toxicity using two species under Good Laboratory Practice (GLP) conditions was proposed to be carried out by Dr. David McCormick as a subcontract to the IIT Research Institute, Chicago. Experiments were designed to conduct preclinical toxicity of 1α-Hydroxyvitamin D_5 using rats and dogs. The studies using male and female CD rats are completed. Only the summary with salient features is presented here. A detailed report is attached in the appendix. The experiments to evaluate the toxicity of vitamin D analog in dogs are underway. The dogs are being held in quarantine, and the study will begin in September. The results will be used to assure safe dose selection for Phase I clinical trials next year.

FOUR-WEEK ORAL (GAVAGE) TOXICITY STUDY OF 1-HYDROXYVITAMIN D_5 IN RATS

Male and female CD rats (ten per gender per group) were dosed once a day for at least 28 consecutive days in order to evaluate the toxicity of the test article, 1-Hydroxyvitamin D_5 ($1D_5$). $1D_5$ was administered as a solution in corn oil by oral gavage at doses of 2.5, 5.0, and 10.0 μg/kg of body weight. A vehicle control group was administered an equivalent volume (5 ml/kg) of corn oil (vehicle). Ten rats/gender/group were necropsied after 28(males) or 29 (females) days of dosing (Day 29 or 30, respectively). Ten additional animals in each of the vehicle control and high-dose (10.0 μg/kg) groups were allowed a 14-day recovery period following dosing to determine the reversibility of any observed toxic effects, and were necropsied on Day 43. All animals were observed for adverse clinical signs, and body weight, body weight gain, and food consumption were measured weekly. Clinical pathology (hematology, including coagulation and red cell morphology, and clinical chemistry) parameters were evaluated at the end of the 28-day treatment period and again after 2 weeks of recovery. All rats were subjected to a gross necropsy, and tissues from control and high-dose animals sacrificed after the 28-day treatment period were processed and examined microscopically. Target tissues examined in rats from the low- and mid-dose groups and in recovery rats consisted of the kidneys and sternum.

No animals died during the study, and no adverse treatment-related clinical signs of toxicity were observed. No treatment-related effects on body weight, weekly or total body weight gain, or food consumption were observed during the study. Treatment-related, statistically significant increases in serum calcium (11.0 ± 0.46 mg/dL control vs. 11.6 ± 0.73 mg/dL high dose $1D_5$) in high-dose males and (11.2 ± 0.29 mg/dL control vs. 11.9 ± 0.35 mg/dL high $1D_5$) in high-dose females and in mid-dose females and in inorganic phosphorus levels (both genders of the high-dose group) were observed. Calcium and phosphorus were not increased in the recovery animals. Other statistically significant increases or decreases were noted for a few hematological and serum chemistry parameters, but were not considered treatment-related. Several other differences in absolute or relative organ weights were also observed, but were not considered treatment-related. No treatment-related gross lesions were observed at necropsy.

The increases in serum calcium seen in the high-dose males and females, and in the mid-dose females, correlated microscopically with statistically significant (p 0.05) increased incidences of mineralization in the kidney (medulla, pelvic epithelium, and/or cortico-medullary junction) of these animals. Mineralization of the renal pelvic epithelium was also present in the mid-dose males, while mineralization also occurred in the kidney of low-dose females. Other treatment-related microscopic changes seen in the kidney of females at all dose levels consisted of the presence of basophilic tubules and tubular dilatation. Degeneration of cartilage was also seen microscopically in the sternum of both genders at all dose levels. All microscopic findings were of minimal to mild severity. At the end of the recovery period, the incidence of renal pelvic epithelial mineralization was still slightly increased in both sexes, while the incidence of basophilic renal tubules and tubule dilation was still increased in the females. The change in the sternum was no longer present in the recovery rats.

In conclusion, administration of 1-Hydroxyvitamin D_5 to male and female rats via oral gavage for four weeks at doses of 2.5, 5.0 and 10.0 μg/kg resulted in increased serum calcium levels in both sexes at the high-

dose level and in females at the mid-dose level. Microscopic lesions consisting primarily of increased renal mineralization were seen in males at the mid and high dose levels and in females at all dose levels. Although a no-effect level was not established in this study, the toxicological significance of microscopic lesions occurring at all dose levels was considered to be minimal because of the minimal severity of the lesions and because these lesions also occur as incidental findings in rodent studies. For further details, see toxicity report in appendix.

Synthesis of 1α-Hydroxyvitamin D_5 under GMP

As proposed in the original application the synthesis of 1α-Hydroxyvitamin D_5 is being carried out by Drs. Robert Moriarty and Raju Penmasta at Conquest Inc. (formerly known as Steroids Ltd.). Dr. Moriarty has synthesized and supplied 1α-hydroxyvitamin D_5 for all our prior studies. As a part of this project, a subcontract to Dr. Moriarty is awarded for him to supply 1 gram of the compound for preclinical toxicity and 1 gram of the analog synthesized under Good Manufacturing Practice (GMP). Dr. Moriarty already synthesized and supplied 1 gram of 1α(OH) D_5 for preclinical toxicity. Experiments described under preclinical toxicity used this newly synthesized compound. The synthesis of D_5-analog under GMP is being carried out. The process involves 12 steps. Following is the summary of the progress of this synthesis prepared by Dr. Robert Moriarty and Dr. Raju Penmasta.

The first five steps require synthesis of sitosterol from stigmasterol. This process does not require GMP processing. The next seven steps have to be carried out under GMP requirements. The first five steps have been completed. Following is the summary of the synthesis.

Step 1: Stigmasterol Tosylate:

Stigmasterol (50 gms) was dissolved in pyridine (175 ml) and cooled in ice-bath to O-5°C. To this was added in several portions Tosyl chloride (43 gms) over a period of 0.5 hrs. The resulting solution was stirred at room temperature in dark for 20 hrs. Progress of the reaction was monitored by TLC (5 % Hexane: Ethyl acetate RF 0.5). The reaction mixture was poured into cold 5% $NaHCO_3$ solution, wherein tosylate precipitated out. The solid was stirred for 15 min, filtered washed with water, and air dried to yield Stigmasterol Tosylate in 64 grams.

1H NMR data: ∂ 0.9- 2.4 (m, skeletal protons), 2.5 (s, Methyl of tosyl group), 4.3 (m), 4.9-5.3 (m), 7.3 (d), and 7.8 (d)

Step 2: Preparation of Stigmasterol methyl ether:

A suspension of stigmasterol tosylate (64 gms), potassium acetate (70 gms), and anhydrous methanol was refluxed for 5 hrs. The reaction was monitored by TLC (RF = 0.7, 5% Hexane: EtOAc). MeOH was evaporated in vacuum, and ether was added and washed with water, 5 % NaHCO3, and brine and dried over sodium sulfate. The solvent was concentrated in vacuum to afford 45 gms of methyl ether as a pale yellow viscous liquid.

1H NMR data : ∂ 0.5-2 (m, skeletal protons) 3.3 (s, OMe); 4.9-5.2 (m).

Step 3: Preparation of sitosterol methyl ether:

A solution of stigmasterol methyl ether (10 gms) in ethyl acetate (250 ml) and 10 % Pd/C (3 gms) was stirred at room temperature under H_2 atmosphere using balloon for three days. The catalyst filtered through celite, and the solvent was removed to afford the sitosterol methyl ether. The yield was 9.5 gms.

1 H NMR: ∂ 0.5- 2 (m, skeletal protons), 3.3 (s, Ome).

Step 4: Preparation of sitosterol acetate:

A solution of sitosterol methyl ether (50 gms) in glacial acetic (1-liter) acid was refluxed with Zinc acetate (65 gms) for 3 hrs. The reaction was monitored by TLC (RF = 0.4, 5 % Hexane: EtOAc). Then the reaction mixture was cooled to room temperature, and water was added. The resulting white precipitate was

filtered, washed with water, and air-dried. Recrystalization from ether:methanol afforded 42 gms as colorless solid sitosterol acetate.

1 H NMR : ∂ 0.5-1.9 (m, skeletal protons), 2.0 (s, COMe), 2.3 (d,), 4.5-4.7 (m,), 5.35 (d).

Step 5: Preparation of 7-Dehydrositosterol :

A suspension of sitosterol (1 gm), anhydrous NaHCO3 (0.9 gms), and dibromontin in hexane (25 ml) was refluxed for 2 hrs. The reaction mixture was cooled to room temperature and filtered, and then the solvent was removed under vacuum. To the reaction flask, THF was added followed by tetrabutyl ammonium bromide (0.061 gms). The solution was stirred at room temperature for 30 minutes. To this reaction mixture was added tetrabutylammonium fluoride (2.92) and pyridine (0.5 ml). Then the reaction mixture was stirred at room temperature for 20 hrs. The crude reaction mixture was transferred to a separating funnel, water layer was removed, washed the organic layer with water, 1 N HCl, water, and then saline. The organic layer was dried and concentrated in vaccuo to afford a dark brown viscous liquid. The crude reaction mixture was purified by column chromatography (silica gel; Ethyl-hexane 1:9 mixture as eluent), to afford 7-dehydrositosterol acetate as a semi-solid.

1H NMR: 0.5- 2.5 (m, skeletal protons), 3.5- 3.7 (m), 5.3- 5.4 (m), 5.5-5.6 (m)

In conclusion: One gram of 1α-hydroxyvitamin D_5 was supplied for preclinical toxicity studies. Currently synthesis of this analog under GMP is being carried out. The synthesis of 1α-hydroxy vitamin D5 involves twelve steps. The first five steps of the synthesis have been completed. There are seven additional steps to make the final product (i.e., 1α-hydroxyvitamin D5). The last five steps of the synthesis involve GMP. Our goal is to make and supply 1 gm of 1α-Hydroxyvitamin D5 under GMP by the end of this year to Dr. T. K. Das Gupta at the University of Illinois at Chicago for scheduled clinical trial.

Plan for the Clinical Trial

The protocol and informed consent form (both in English and Spanish) of the Phase 1/2 clinical trial are currently under review by both the U.S. Army human research Regulatory Compliance and Quality review committee (Ms. Catherine A. Smith, Human Subjects Protection Specialist) and the UIC institutional review board (Dr. Eric Gislason, Vice-Chancellor). Once the protocol is approved by these two bodies and the preclinical toxicity study results are final, the FDA application (IND #56509) will be completed so that the current clinical hold pending these studies will be withdrawn. Once the FDA's approval is received, patient accrual will be started.

Key Research Accomplishments

- We have completed preclinical the toxicity study in male and female rats under GLP. Males and females were given 1-10 μg/kg body weight 1α(OH)D_5 by oral gavage for 28 consecutive days.
- 1α(OH)D_5 showed no serious toxic effect. No animals died during the study, and no adverse treatment-related clinical signs of toxicity were observed. No treatment-related effects on body weight, weekly or total body weight gain, or food consumption were observed during the study.
- Increased serum calcium levels in both sexes at the high dose level and in females at the mid dose level. Microscopic lesions consisting primarily of increased renal mineralization were seen in males at the mid and high dose levels and in females at all dose levels. Although a no-effect level was not established in this study, the toxicological significance of microscopic lesions occurring at all dose levels was considered to be minimal because of the minimal severity of the lesions and because these lesions also occur as incidental findings in rodent studies.
- Effect of 1α(OH)D_5 was reversible. Within two weeks after discontinuation of the treatment, serum calcium levels and renal mineralization lesions reached the same levels as the control group.

- We began the synthesis of $1\alpha(OH)D_5$ according to GMP for the future phases I clinical trials. We completed 5 steps in the synthesis of the compound.
- The effect of in vitro $1\alpha(OH)D_5$ was observed in normal breast tissues, fibroadenomas, and breast carcinomas obtained from women with confirmed diagnosis of the disease. Our results show that
  - Normal breast tissue retains the original alveolar and ductal structures when incubated in the culture medium used in this study. Breast epithelial cells appear to be normal and alive for 72 hours. All epithelial cells show VDR expression. Many appear to be proliferating, as evident from Ki-67 staining. $1\alpha(OH)D_5$ (1 μM) treatment shows no toxic effect on the breast epithelial cells; all alveolar and ductal structures are preserved. $1\alpha(OH)D_5$ has no effect on cell proliferation.
  - Breast fibroadenomas retain normal structures in in vitro culture for 72 hours. Following incubation with $1\alpha(OH)D_5$, many alveolar structures show apoptotic or degenerative epithelial cells. The cells unaffected by $1\alpha(OH)D_5$ show high expression of VDR.
  - Breast carcinomas treated with $1\alpha(OH)D_5$ show a significant number of cells undergoing pyknosis or apoptosis.

Reportable outcomes

Publication:

1) Lazzaro G., Agadir A., Qing W., Poria M., **Mehta R.R.**, Moriarty R.M., Zhang X., Mehta R.G. Induction of differentiation by $1\alpha(OH)D_5$ in T47D human breast cancer cells and its interaction with vitamin D receptor. Eur. J. Cancer, 36, 780-786, 2000 (see attached)

Conclusions:

$1\alpha(OH)D_5$ selectively influences the breast carcinoma cells. It has no effect on normal breast epithelial cells. Our preclinical studies in rats suggest that $1\alpha(OH)D_5$ has no serious toxic effect. At high dose (5-10 μg/kg body weight), $1\alpha(OH)D_5$ showed increased serum calcium levels and some renal mineralization; however, this effect was reversible.

References:

1. Gudas LJ, Sporn MB, Roberts AB. Cellular biology and biochemistry of the retinoids. In: Sporn MB, Roberts AB, Goodman DS (eds.), The Retinoids, 2nd ed. New York, Raven Press, 1994, pp 443-520.

2. Deluca HF and Ostrem V. The relationship between the vitamin D system and cancer. Adv. Exp. Biol. Med. 206:413-29, 1986.

3. Yamazaki T, Yoshiki S, and Suda T. Differentiation of mouse myseloid leukemia cells induced by vitamin D3. Proc. Natl. Acad Sciences 78:4990, 1981.

4. Koga M, Eisman JA, and Sutherland RL. Regulation of epidermal growth factor receptor levels by 1,25-dihydroxy vitamin D3, in human breast cancer cells. Cancer Res. 48: 2734-39, 1988.

5. Taoka T, Collins ED, Irino S, and Norman AW. 1, 25(OH)2-vitamin D3 mediated changes in m-RNA for c-myc and 1,25 (OH)2 vitamin D3 receptor in Hl-60 cells and related sub clones. Molec. Cell Endocrinol 95:77-82, 1995.

6. Coloston KW, McKay AG, James SY, Binderup S, and Coombes RC. EB1089: A new vitamin D analogue that inhibits the growth of breast cancer cells in vivo and in vitro. Biochem. Pharmacol. 44:2273-80, 1992.

7. Abe-Hoshimoto, Kikuchi T, Mastumoto T, Nishii Y, Ogata T, and Ikeda K. Antitumor effect of 22-oxa-calcitrol, a new noncalcemic analog of calcitrol in athymic mice implanted with human breast carcinoma and its synergism with tamoxifen. Cancer Res. 53:2534-2537, 1993.

8. Wijnggsarden T, Pols HAP, Burrman CJ, van den Bemd, JCM, Dorssers LCJ, Birkenhager JC, and van Leeuwen JPTM. Inhibition of breast cancer cell growth by combined treatment with vitamin D3 analogs and tamoxifen. Cancer Res. 54:5711-17, 1994.

9. Anzano MA, Smith JM, Uskokovic MR, Peer CW, Mullen LT, Letterio JJ, Welsh MC, Shrader MW, Logsdon DL, Driver CL. 1-alpha,25-Dihydroxy-16-ene-23-yne-26,27-hexafluorocholecalcifer (RO24-5531), 1 new deltanoid (vitamin D analog) for prevention of breast cancer in the rat. *Cancer Res.* 54:1653-56, 1994.

10. Elstner E, Linker-Israeli M, Said J, Umiel T, Sven V, Shintaku P, Heber D, Binderup L, Uskokovic M, and Koemer P. 20-epi-vitamin D3 analogues: A new class of potent inhibitors of proliferation and inducers of differentiation of human breast cancer cell lines. *Cancer Res.* 55:2822-2830, 1995.

11. Brenner RV, Shabahang M, Schumaker LM, Nauta RJ, Uskokovic MR, Evana SR, Buras RR. The anti-proliferative effect of vitamin D analogs on MCF-7 human breast cancer cells. Cancer Lett. 9285:77-82, 1995.

12. James SY, Mckay AG, Coloston KW. Vitamin D derivatives in combination with 9-Cis retinoic acid promote active cell death in breast cancer cells. J. Mol. Endocrinol. 14: 391-394, 1995.

13. Koike M, Elstner E, Campbell M.J., Asou C, Uskokovic M, Tsurouks N, and Koeffler P. 19-nor-Hexafluoride analog of vitamin D3: A novel class of potent inhibitors of proliferation of human breast cell lines. Cancer Res. 57, 4545-4550, 1997.

14. Lopez-Boado YS, Puente XS, Alvarez S, Tolivia J, Binderup L, Lopez-Otin C. Growth inhibition of human breast cancer cells by 1,25-dihydroxyvitamin D3 is accompanied by induction of Apolipoprotein D expression. Cancer Res. 57, 4091-4097, 1997.

15. Mehta RG, Moriarty R, Mehta RR, Penmasta R, Lazzaro G, Constantinou AI, Hawthorne M. Prevention of carcinogen-induced preneoplastic mammary lesions in mice in 1α hydroxyvitamin D_5. JNCI 89:212-8, 1997.

16. Mehta RR, Bratescu L, Graves JM, and Mehta RG. Differentiation of human breast carcinoma cell line UISO-BCA-4 by a novel vitamin D analog: 1α(OH)D5.Int. J. Oncology, 16, 65-73, 2000.

17. Lazzaro G., Agadir A., Qing W., Poria M., **Mehta R R**. T. K. Das Gupta, Moriarty R.M., Zhang X, Mehta R.G. Induction of differentiation by 1a(OH)D5 in T47D human breast cancer cells and its interaction with vitamin D receptor. Eur. J. Cancer, 36, 780-786, 2000

18. Zutter MM, Santoro SA, Painter AS, Tsung YL, Gafford. The human alpha 2 integrin gene promoter. Identification of positive and negative regulatory elements important for cell-type and developmentally restricted gene expression. Biol Chem 7;269(1):463-9 1994

Appendices

Plates 1-6

Plate 1. Histopathology of nonmalignant breast tissues incubated in culture medium alone, in medium containing $1\alpha(OH)D_5$ or $1,25(OH)2D_3$ at 37°C for 0-72 hours.

Plate 2. Ki-67 immunostaining in nonmalignant breast tissue incubated in the culture medium alone, in medium containing $1\alpha(OH)D_5$ or $1,25(OH)2D_3$ at 37°C for 0-72 hours.

Plate 3. Immunostaining for intracellular casein nonmalignant breast tissue incubated in the culture medium alone, in medium containing $1\alpha(OH)D_5$ or $1,25(OH)2D_3$ at 37°C for 0-72 hours.

Plate 4. Immunostaining for VDR nonmalignant breast tissue incubated in the culture medium alone, in medium containing $1\alpha(OH)D_5$ or $1,25(OH)2D_3$ at 37°C for 0-72 hours.

Plate 5. Histopathology of breast fibroadenoma nonmalignant breast tissue incubated in the culture medium alone, in medium containing $1\alpha(OH)D_5$ or $1,25(OH)2D_3$ at 37°C for 0-72 hours. Arrow shows alveolar structure with degenerated epithelial cells.

Immunostaining for Ki-67 in fibroadenoma incubated in the culture medium alone (C), in medium containing $1\alpha(OH)D_5$ (D) at 37°C for 72 hours.

Immunostaining for VDR in fibroadenoma incubated in the culture medium alone (E), in medium containing $1\alpha(OH)D_5$ (F) at 37°C for 72 hours.

Plate 6. Histopathology of breast carcinoma incubated in the culture medium alone (E), in medium containing 1 μM $1\alpha(OH)D_5$ (F) at 37°C for 48 hours. Arrow shows apoptotic cells.

Figures 1-4

Figure 1. Effect of $1\alpha(OH)D_5$ on in vivo growth of MDA-MB-231 cells transplanted into athymic mice. Animals were given $1\alpha(OH)D_5$ (25 μg/kg diet) mixed in the diet. Each group consisted of five animals. Data represent mean + SE of group.

Figure 2. Effect of $1\alpha(OH)D_5$ on in vitro growth of MDA-MB-231 cells.

Figure 3. Effect of $1\alpha(OH)D_5$ on in vitro growth of S-30 (ER-transfected MDA-MB-231) cells.

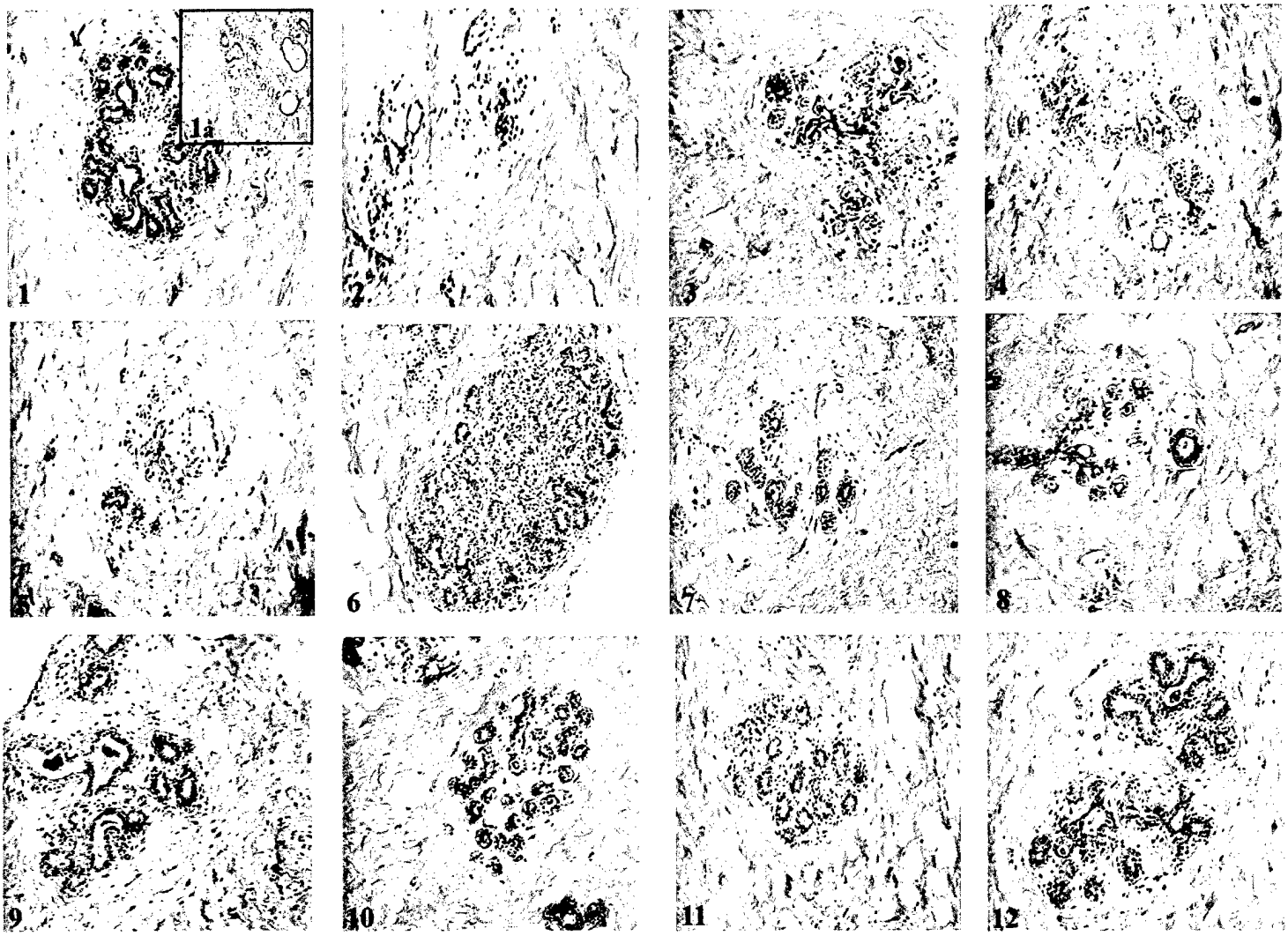
Figure 4a. Alpha2 integrin expression in MDA-MB-231 and S-30 (ER-transfected MDA-MB-231) breast carcinoma cell line.

Figure 4b. Effect of $1\alpha(OH)D_5$ on alpha2 expression on MDA-MB-231 and ER-transfected MDA-MB-231 (S-30) cell lines.

A detailed preclinical toxicity report

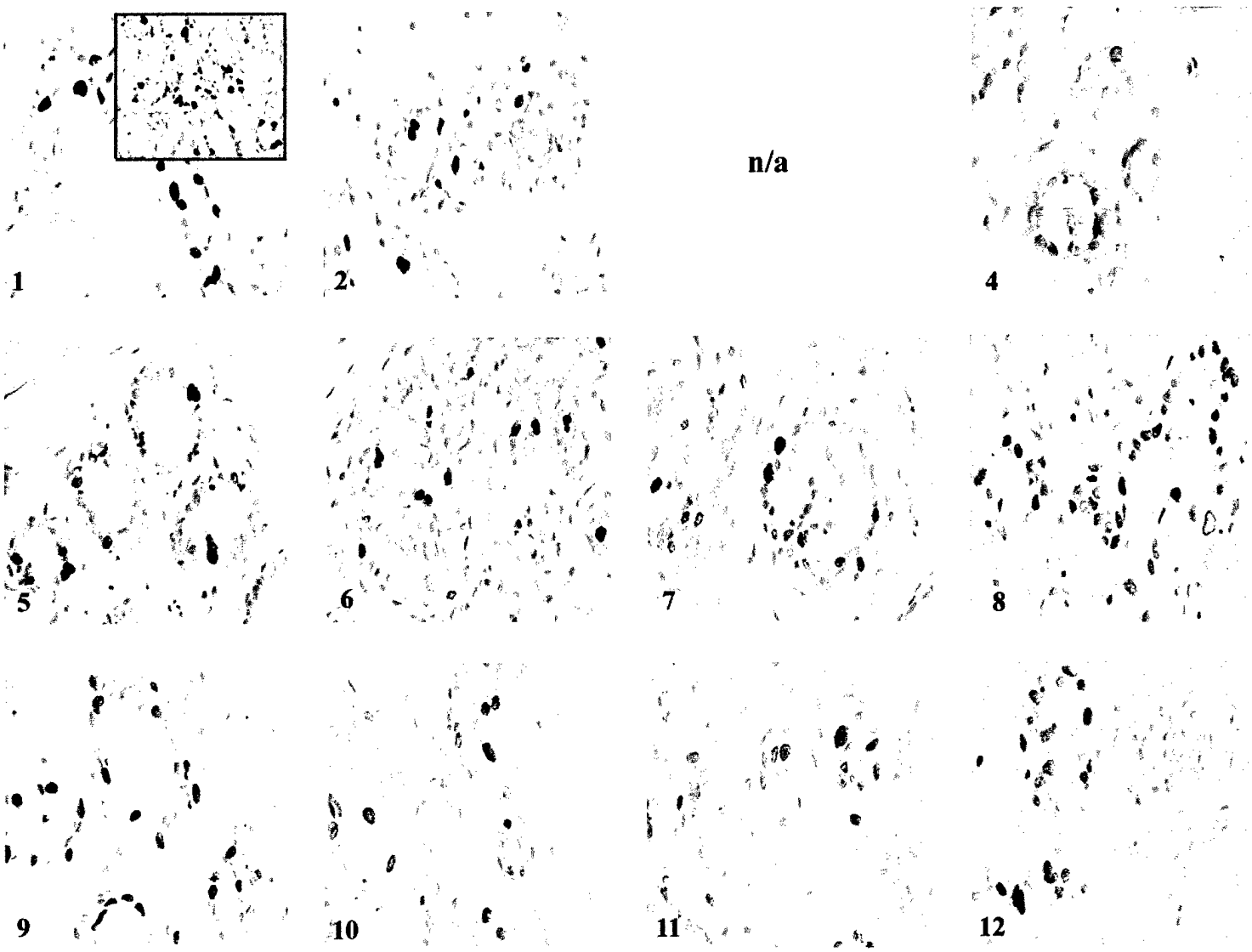
Manuscript, Eur. J. Cancer Eur. J. Cancer, 36, 780-786, 2000

Plate 1. Histopathology of nonmalignant breast tissues incubated in culture medium alone, in medium containing $1\alpha(OH)D_5$ or $1,25(OH)2D_3$ at 37°C for 0-72 hours.



1. Control 24 hrs. (1a Time 0)
2. $1.25(OH)_2D_3$ 24 hrs.
3. 10^{-7}M $1\alpha(OH)D_5$ 24 hrs.
4. 10^{-6}M $1\alpha(OH)D_5$ 24 hrs.
5. Control 48 hrs.
6. 1.25(OH) D 48 hrs.
7. 10^{-7}M $1\alpha(OH)D_5$ 48 hrs.
8. 10^{-6}M $1\alpha(OH)D_5$ 48 hrs.
9. Control 72 hrs.
10. $1.25(OH)_2D_3$ 72 hrs.
11. 10^{-7}M $1\alpha(OH)D_5$ 72 hrs.
12. 10^{-6}M $1\alpha(OH)D_5$ 72 hrs.

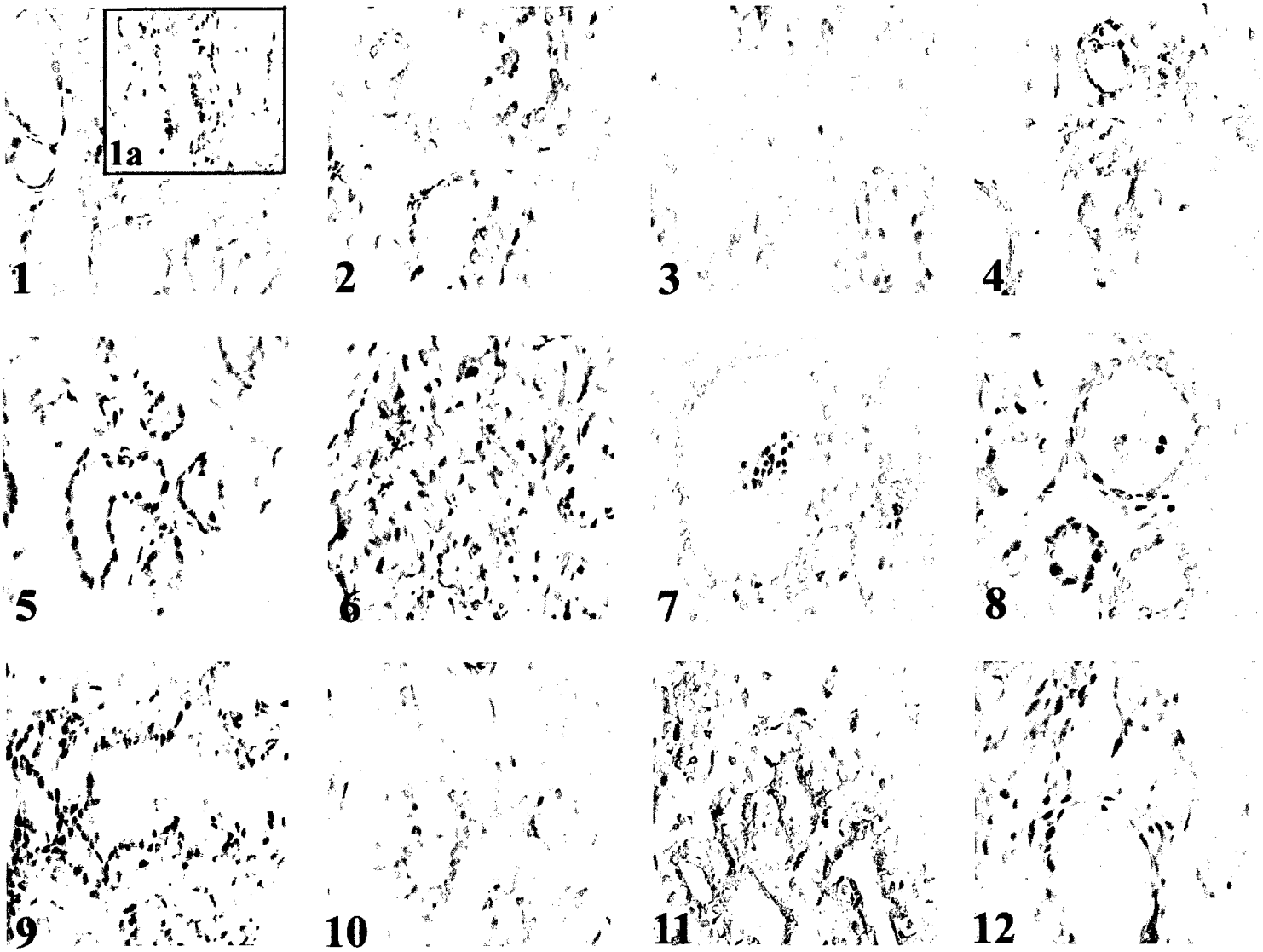
Plate 2. Ki-67 immunostaining in nonmalignant breast tissue incubated in the culture medium alone, in medium containing $1\alpha(OH)D_5$ or $1,25(OH)2D_3$ at 37°C for 0-72 hours.



KI 67 stain

1. Control 24 hrs. (1a Time 0)
2. $1.25(OH)_2D_3$ 24 hrs.
3. 10^{-7}M 1α(OH)D_5 24 hrs. (photo not shown)
4. 10^{-6}M 1α(OH)D_5 24 hrs.
5. Control 48 hrs.
6. 1.25(OH) D 48 hrs.
7. 10^{-7}M 1α(OH)D_5 48 hrs.
8. 10^{-6}M 1α(OH)D_5 48 hrs.
9. Control 72 hrs.
10. $1.25(OH)_2D_3$ 72 hrs.
11. 10^{-7}M 1α(OH)D_5 72 hrs.
12. 10^{-6}M 1α(OH)D_5 72 hrs.

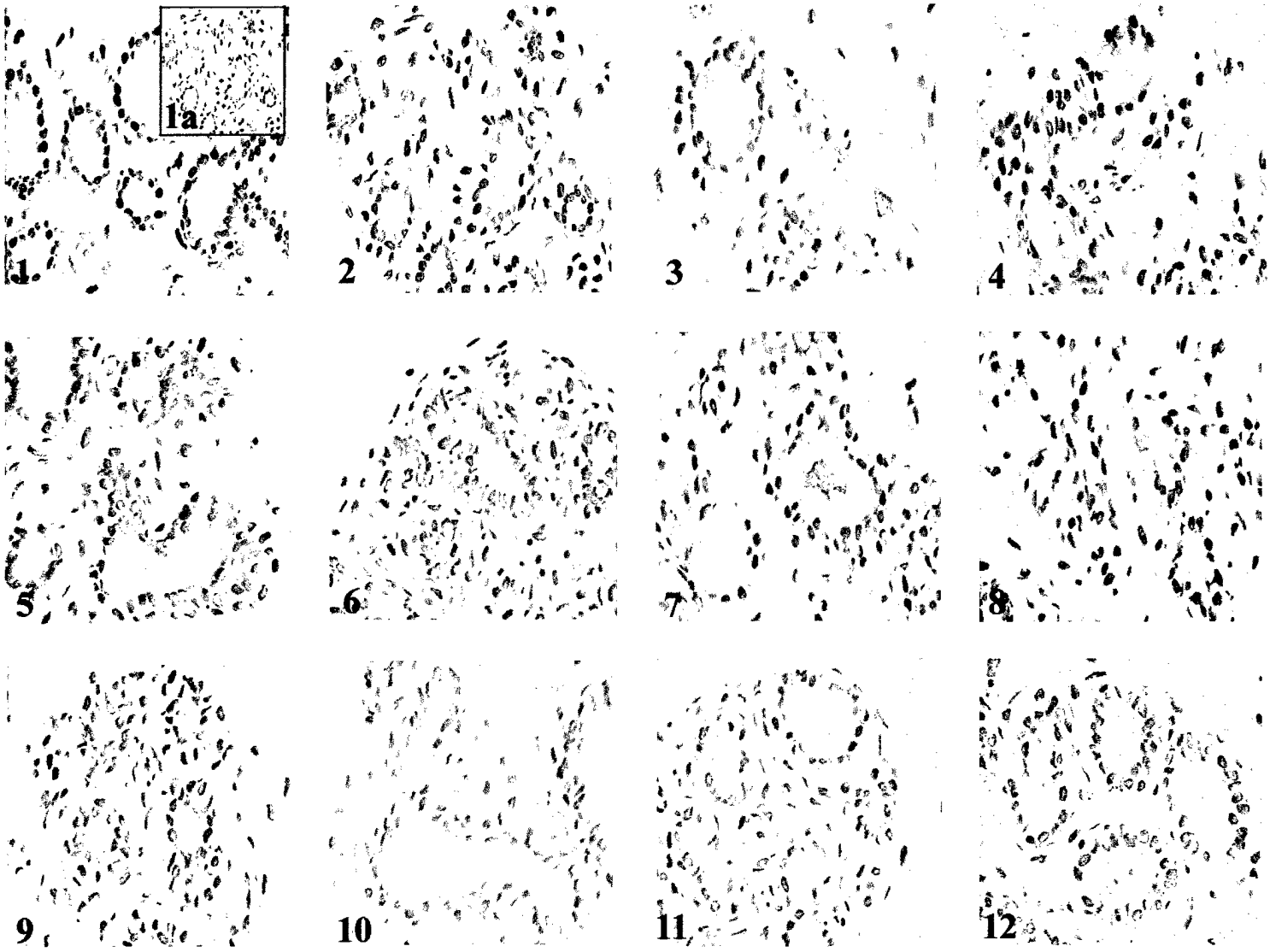
Plate 3. Immunostaining for intracellular casein nonmalignant breast tissue incubated in the culture medium alone, in medium containing $1\alpha(OH)D_5$ or $1,25(OH)2D_3$ at 37°C for 0-72 hours.



Casein stain

1. Control 24 hrs. (1a Time 0)
2. $1.25(OH)_2D_3$ 24 hrs.
3. 10^{-7}M $1\alpha(OH)D_5$ 24 hrs.
4. 10^{-6}M $1\alpha(OH)D_5$ 24 hrs.
5. Control 48 hrs.
6. 1.25(OH) D 48 hrs.
7. 10^{-7}M $1\alpha(OH)D_5$ 48 hrs.
8. 10^{-6}M $1\alpha(OH)D_5$ 48 hrs.
9. Control 72 hrs.
10. $1.25(OH)_2D_3$ 72 hrs.
11. 10^{-7}M $1\alpha(OH)D_5$ 72 hrs.
12. 10^{-6}M $1\alpha(OH)D_5$ 72 hrs.

Plate 4. Immunostaining for VDR nonmalignant breast tissue incubated in the culture medium alone, in medium containing $1\alpha(OH)D_5$ or $1,25(OH)2D_3$ at 37°C for 0-72 hours.



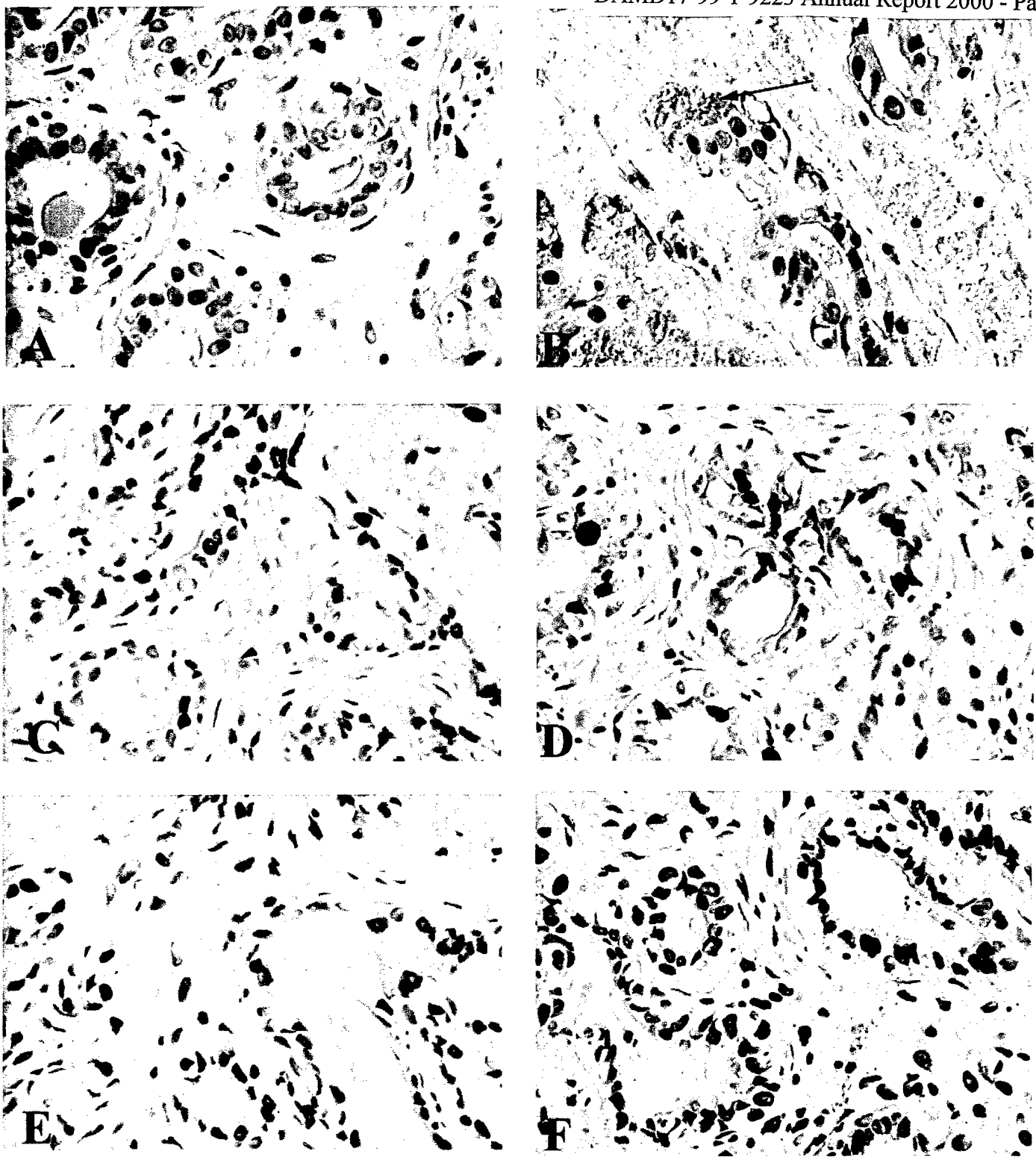
VDR stain

1. Control 24 hrs. (1a Time 0)
2. $1.25(OH)_2D_3$ 24 hrs.
3. 10^{-7}M $1\alpha(OH)D_5$ 24 hrs.
4. 10^{-6}M $1\alpha(OH)D_5$ 24 hrs.
5. Control 48 hrs.
6. 1.25(OH) D 48 hrs.
7. 10^{-7}M $1\alpha(OH)D_5$ 48 hrs.
8. 10^{-6}M $1\alpha(OH)D_5$ 48 hrs.
9. Control 72 hrs.
10. $1.25(OH)_2D_3$ 72 hrs.
11. 10^{-7}M $1\alpha(OH)D_5$ 72 hrs.
12. 10^{-6}M $1\alpha(OH)D_5$ 72 hrs.

Plate 5. Histopathology of breast fibroadenoma nonmalignant breast tissue incubated in the culture medium alone, in medium containing $1\alpha(OH)D_5$ or $1,25(OH)2D_3$ at 37°C for 0-72 hours. Arrow shows alveolar structure with degenerated epithelial cells.

Immunostaining for Ki-67 in fibroadenoma incubated in the culture medium alone (C), in medium containing $1\alpha(OH)D_5$ (D) at 37°C for 72 hours.

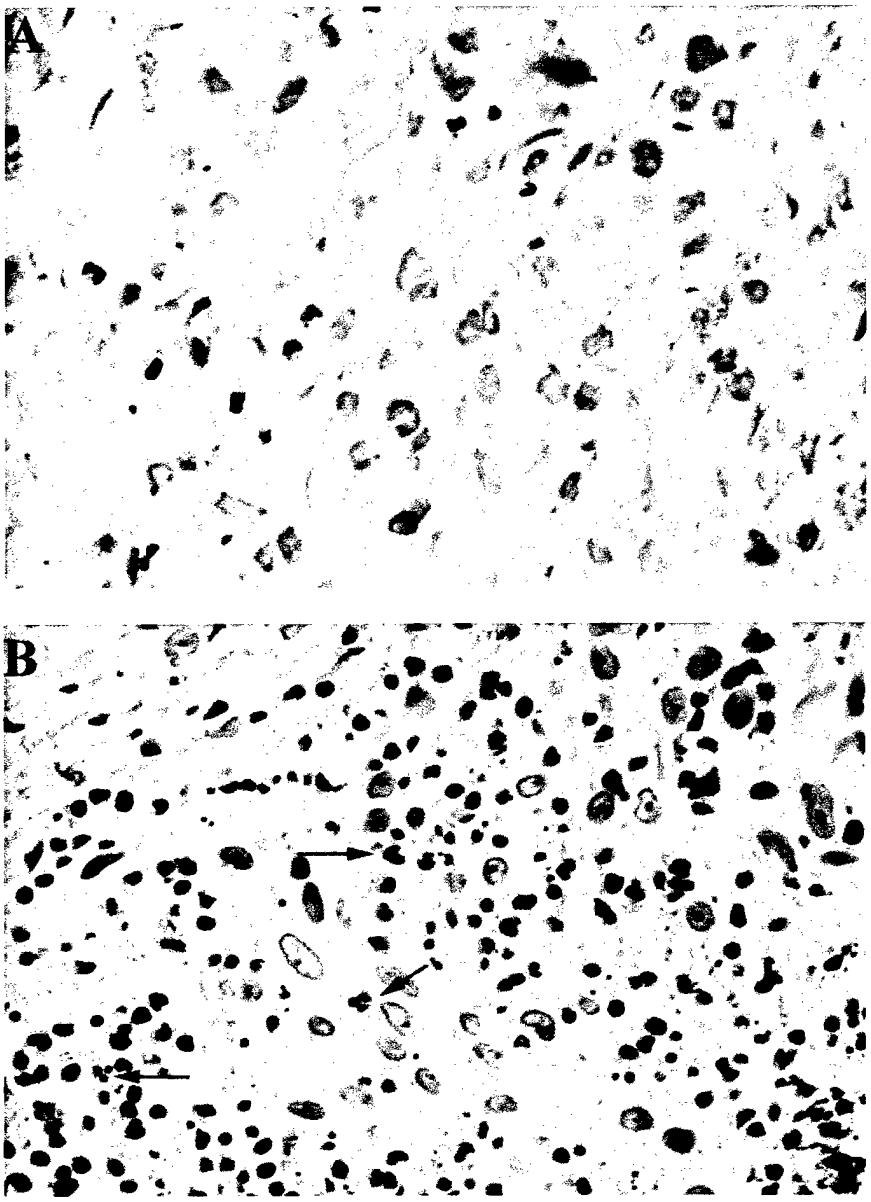
Immunostaining for VDR in fibroadenoma incubated in the culture medium alone (E), in medium containing $1\alpha(OH)D_5$ (F) at 37°C for 72 hours.



Fibroadenoma

A. Control
B. $1\alpha(OH)D_5$
C. KI-67 control
D. KI-67 $1\alpha(OH)D_5$
E. VDR control
F. VDR $1\alpha(OH)D_5$

Plate 6. Histopathology of breast carcinoma incubated in the culture medium alone (E), in medium containing 1 μM $1\alpha(OH)D_5$ (F) at 37°C for 48 hours. Arrow shows apoptotic cells.



Breast Carcinoma

A. Control

B. $1\alpha(OH)D_5$

Figure 1. Effect of $1\alpha(OH)D_5$ on in vivo growth of MDA-MB-231 cells transplanted into athymic mice. Animals were given $1\alpha(OH)D_5$ (25 μg/kg diet) mixed in the diet. Each group consisted of five animals. Data represent mean + SE of group.

Effect of 1α(OH)D_5 supplemented in the diet on the growth of MDA-MB-231 cells transplanted in to athymic mice

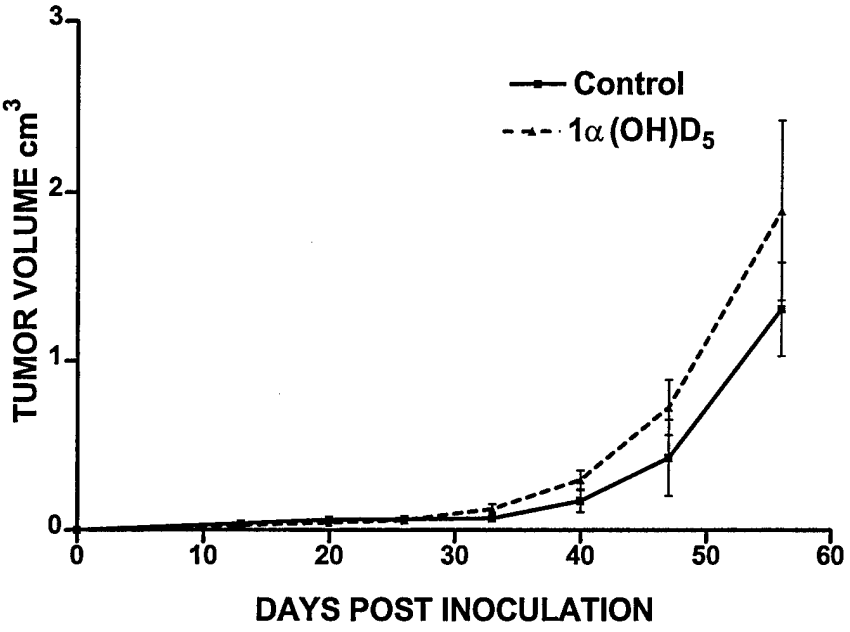


Figure 2. Effect of $1\alpha(OH)D_5$ on in vitro growth of MDA-MB-231 cells.

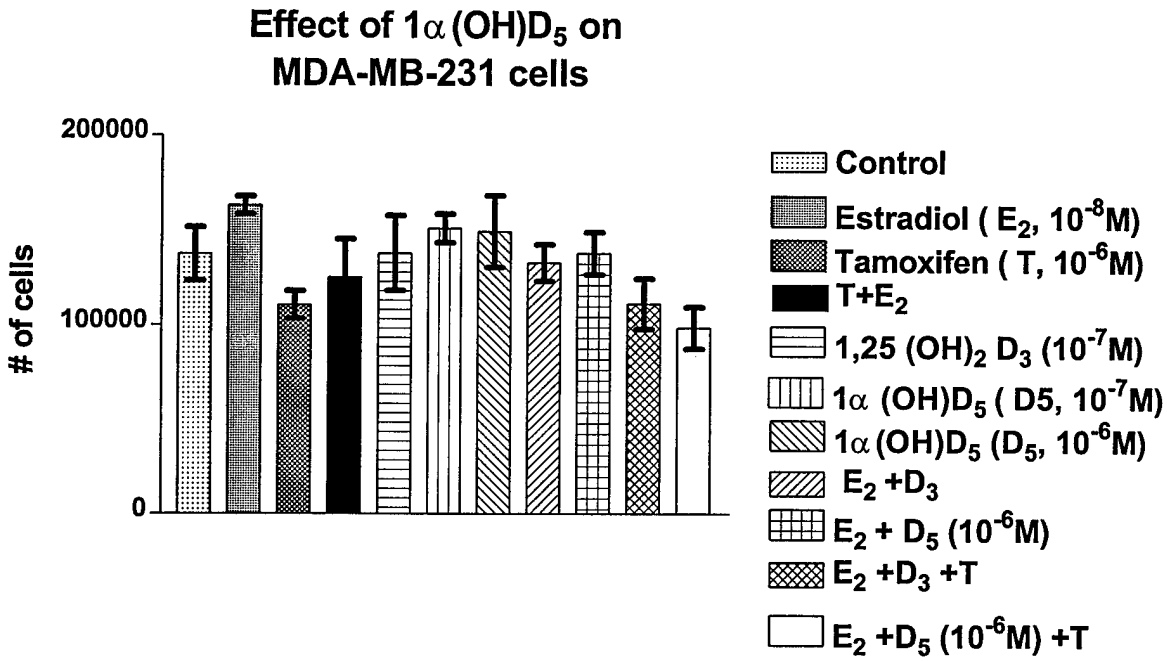

Effect of 1α(OH)D5 on
MDA-MB-231 cells
# of cells
200000
100000
0
Control
Estradiol (E2, 10-8M)
Tamoxifen (T, 10-6M)
T+E2
1,25 (OH)2 D3 (10-7M)
1α (OH)D5 (D5, 10-7M)
1α(OH)D5 (D5, 10-6M)
E2 +D3
E2 + D5 (10-6M)
E2 +D3 +T
E2 +D5 (10-6M) +T

Figure 3. Effect of $1\alpha(OH)D_5$ on in vitro growth of S-30 (ER-transfected MDA-MB-231) cells.

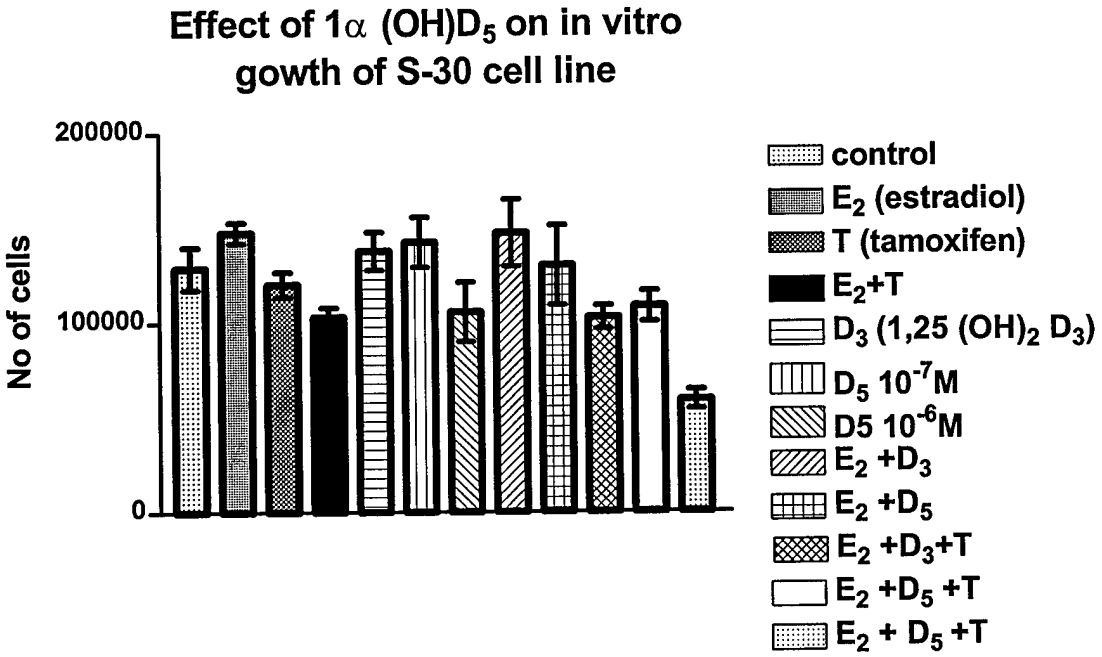

Effect of 1α (OH)D5 on in vitro gowth of S-30 cell line
No of cells
200000
100000
0
control
E2 (estradiol)
T (tamoxifen)
E2+T
D3 (1,25 (OH)2 D3)
D5 10-7M
D5 10-6M
E2 +D3
E2 +D5
E2 +D3+T
E2 +D5 +T
E2 + D5 +T

Figure 4a. Alpha2 integrin expression in MDA-MB-231 and S-30 (ER-transfected MDA-MB-231) breast carcinoma cell line.

Figure 4b. Effect of $1\alpha(OH)D_5$ on alpha2 expression on MDA-MB-231 and ER-transfected MDA-MB-231 (S-30) cell lines.

Figure 4a.

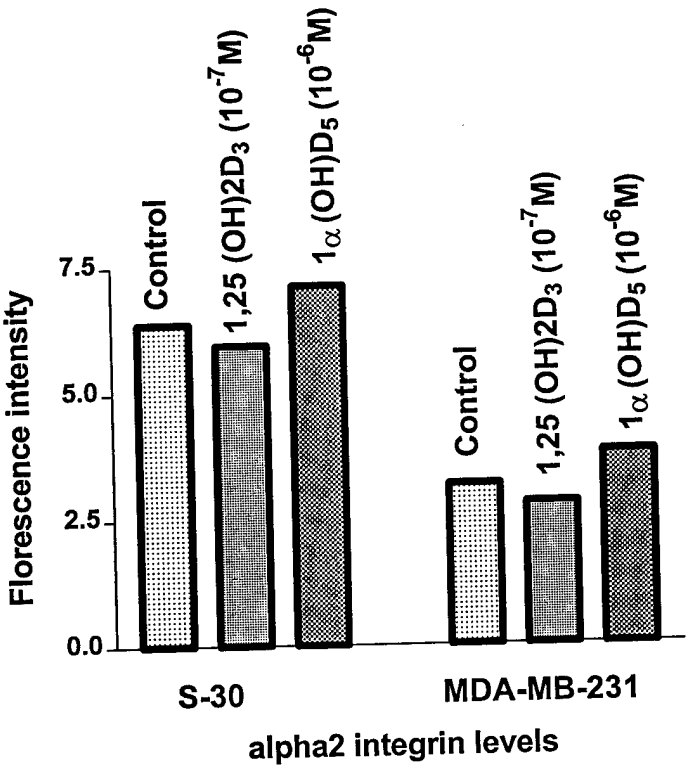
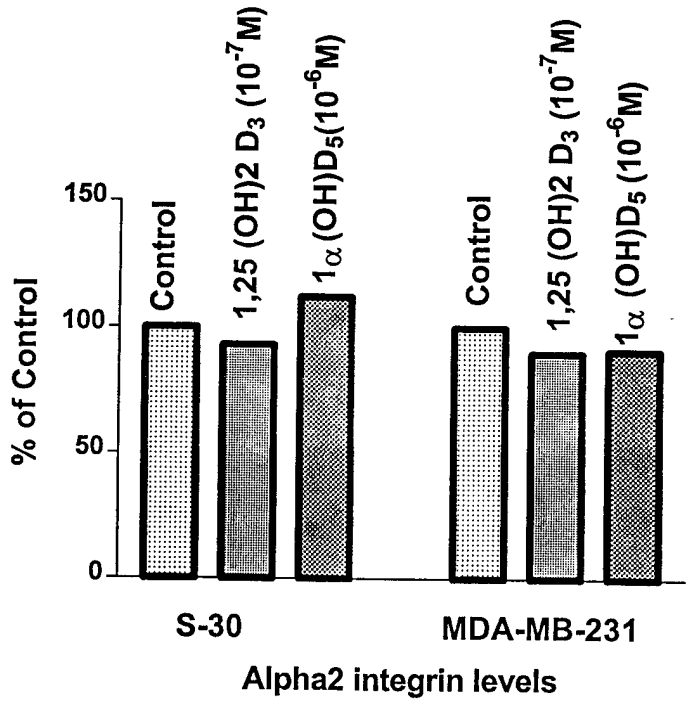


Figure 4b



Detailed preclinical toxicity report

FOUR-WEEK ORAL (GAVAGE) TOXICITY STUDY OF 1α-HYDROXYVITAMIN D_5 IN RATS

TABLE 1

Abbreviations

ALB	- albumin (grams / deciliter serum)
A/G RATIO	- albumin / globulin ratio
ALP	- alkaline phosphatase (international units / liter serum)
ALT	- alanine aminotransferase (international units / liter serum)
AST	- aspartate aminotransferase (international units / liter serum)
BASO	- basophils (absolute: thousands of cells / cubic millimeter blood; relative: percent leukocytes counted)
BUN	- blood urea nitrogen (milligrams nitrogen / deciliter serum)
CA	- calcium (milligrams / deciliter serum)
CHOL	- cholesterol (milligrams / deciliter serum)
CL	- chloride (milliequivalents / liter serum)
CREA	- creatinine (milligrams / deciliter serum)
dL	- deciliter
EOSIN	- eosinophils (absolute: thousands of cells / cubic millimeter blood; relative: percent leukocytes counted)
F	- female
g	- grams
GLOB	- globulin (grams / deciliter serum)
GLU	- glucose (milligrams / deciliter serum)
HCT	- hematocrit (percent)
HGB	- hemoglobin (grams / deciliter blood)
IMM NEU	- immature neutrophils (absolute: thousands of cells / cubic millimeter blood; relative: percent leukocytes counted)
IU	- international units
K	- potassium (milliequivalents / liter serum)
kg	- kilograms
L	- liter
LYMPH	- lymphocytes (absolute: thousands of cells / cubic millimeter blood; relative: percent leukocytes counted)
M	- male
m^2	- square meters
MAT NEU	- mature neutrophils (absolute: thousands of cells / cubic millimeter blood; relative: percent leukocytes counted)
MCH	- mean corpuscular hemoglobin (picograms)
MCHC	- mean corpuscular hemoglobin concentration (percent)
MCV	- mean corpuscular volume (fl=femtoliter; 10^{-15} liter, equivalent to a cubic micron)
mg	- milligrams
mmol	- millimoles
MONO	- monocytes (absolute: thousands of cells / cubic millimeter blood; relative: percent leukocytes counted)
NA	- sodium (milliequivalents / liter serum)
NRBC	- nucleated red blood cells (number / 100 white blood cells)
PLT	- platelet count (thousands / cubic millimeter blood)
RBC	- red blood cell count (millions of cells / cubic millimeter blood)
RETABS	- absolute reticulocyte count (thousands / cubic millimeter blood)
RETPC	- relative reticulocyte count (percent of total erythrocyte count)
SD	- standard deviation
TBIL	- total bilirubin (milligrams / deciliter serum)
TP	- total protein (grams protein / deciliter serum)
TG	- triglycerides (milligrams / deciliter serum)
WBC	- white blood cell count (thousands of cells / cubic millimeter blood); corrected for nucleated red blood cells

FOUR-WEEK ORAL (GAVAGE) TOXICITY STUDY OF 1α-HYDROXYVITAMIN D_5 IN RATS

TABLE 2

SUMMARY OF OBSERVATION FREQUENCY

STUDY: 1209SN1 SEX: MALE

DOSE:(mg/kg)	0	.0025	.005	.01
GROUP:	1-M	2-M	3-M	4-M
Terminal Sacrifice	20	10	10	20
Normal	20	10	10	20
Redness around the eyes	0	0	0	1
Red nasal discharge	2	0	0	0
Redness around nose fur	2	0	0	1
Alopecia	1	0	1	0
Broken/misaligned teeth	0	0	0	1
Total Number of Animals	20	10	10	20

TABLE 2 (cont.)

SUMMARY OF OBSERVATION FREQUENCY

STUDY: 1209SN1 SEX: FEMALE

DOSE:(mg/kg)	0	.0025	.005	.01
GROUP:	1-F	2-F	3-F	4-F
Terminal Sacrifice	20	10	10	20
Normal	20	10	10	20
Chromodacryorrhea	0	0	0	1
Redness around the eyes	0	0	0	2
Red nasal discharge	0	0	0	1
Redness around nose fur	0	0	0	2
Alopecia	1	2	0	0
Ocular opacity	2	0	0	0
Total Number of Animals	20	10	10	20

TABLE 3

SUMMARY OF BODY WEIGHTS (Grams)

STUDY: 1209SN1 SEX: MALE

PERIOD	DOSE: (mg/kg) GROUP:	0 1-M	.0025 2-M	.005 3-M	.01 4-M
DAY 0	MEAN	263	258	258	263
	S.D.	11.8	8.0	9.2	10.2
	N	20	10	10	20
DAY 7	MEAN	304	296	304	306
	S.D.	15.6	13.0	13.2	13.5
	N	20	10	10	20
DAY 14	MEAN	349	336	350	349
	S.D.	16.8	18.4	20.5	19.2
	N	20	10	10	20
DAY 21	MEAN	391	375	389	391
	S.D.	18.3	25.4	29.8	23.5
	N	20	10	10	20
DAY 28	MEAN	426	406	426	425
	S.D.	21.7	31.1	34.7	28.9
	N	20	10	10	20
DAY 35	MEAN	431	--	--	437
	S.D.	20.4	--	--	37.0
	N	10	0	0	10
DAY 42	MEAN	457	--	--	465
	S.D.	21.5	--	--	37.4
	N	10	0	0	10

* P less than .05
** P less than .01
-- = Data Unavailable

Analysis of Variance using DUNNETT'S Procedure

TABLE 3 (cont.)

SUMMARY OF BODY WEIGHTS (Grams)

STUDY: 1209SN1 SEX: FEMALE

PERIOD	DOSE: (mg/kg) GROUP:	0 1-F	.0025 2-F	.005 3-F	.01 4-F
DAY 0	MEAN	196	188	189	195
	S.D.	10.8	6.7	8.0	10.4
	N	20	10	10	20
DAY 7	MEAN	210	202	203	212
	S.D.	11.0	6.5	8.8	13.2
	N	20	10	10	20
DAY 14	MEAN	228	220	222	229
	S.D.	13.0	9.5	9.4	15.0
	N	20	10	10	20
DAY 21	MEAN	244	234	236	243
	S.D.	15.0	10.2	8.0	15.9
	N	20	10	10	20
DAY 28	MEAN	256	244	249	253
	S.D.	16.5	12.1	8.7	17.3
	N	20	10	10	20
DAY 35	MEAN	253	--	--	263
	S.D.	16.1	--	--	20.0
	N	10	0	0	10
DAY 42	MEAN	261	--	--	271
	S.D.	15.6	--	--	20.2
	N	10	0	0	10

* P less than .05
** P less than .01
-- = Data Unavailable

Analysis of Variance using DUNNETT'S Procedure

TABLE 4

SUMMARY OF WEIGHT GAINS (Grams)

STUDY: 1209SN1 SEX: MALE

PERIOD	DOSE: (mg/kg) GROUP:	0 1-M	.0025 2-M	.005 3-M	.01 4-M
DAY 7	MEAN	41	38	46	42
	S.D.	8.0	7.9	9.9	6.9
	N	20	10	10	20
DAY 14	MEAN	45	41	46	43
	S.D.	5.4	8.4	8.8	8.1
	N	20	10	10	20
DAY 21	MEAN	42	39	39	42
	S.D.	5.8	9.0	10.0	7.3
	N	20	10	10	20
DAY 28	MEAN	35	31	38	34
	S.D.	5.6	6.3	6.7	8.1
	N	20	10	10	20
TOTAL GAIN	MEAN	163	148	169	161
	S.D.	14.8	28.1	29.4	24.5
	N	20	10	10	20

* P less than .05
** P less than .01

Analysis of Variance using DUNNETT'S Procedure

TABLE 4 (cont.)

SUMMARY OF WEIGHT GAINS (Grams)

STUDY: 1209SN1 SEX: FEMALE

PERIOD	DOSE: (mg/kg) / GROUP:	0 1-F	.0025 2-F	.005 3-F	.01 4-F
DAY 7	MEAN	15	14	14	17
	S.D.	6.7	5.4	4.3	4.6
	N	20	10	10	20
DAY 14	MEAN	17	17	19	17
	S.D.	4.6	5.6	4.3	3.7
	N	20	10	10	20
DAY 21	MEAN	16	14	14	14
	S.D.	4.3	3.6	3.9	2.7
	N	20	10	10	20
DAY 28	MEAN	12	10	13	10
	S.D.	4.5	3.2	3.9	3.2
	N	20	10	10	20
TOTAL GAIN	MEAN	60	56	60	58
	S.D.	11.2	12.6	6.6	9.7
	N	20	10	10	20

* P less than .05
** P less than .01

Analysis of Variance using DUNNETT'S Procedure

TABLE 4 (cont.)

SUMMARY OF WEIGHT GAINS (Grams)

STUDY: 1209SN1 SEX: MALE

PERIOD	DOSE: (mg/kg) GROUP:	0 1-M	.0025 2-M	.005 3-M	.01 4-M
DAY 7	MEAN	41	38	46	42
	S.D.	8.0	7.9	9.9	6.9
	N	20	10	10	20
DAY 14	MEAN	45	41	46	43
	S.D.	5.4	8.4	8.8	8.1
	N	20	10	10	20
DAY 21	MEAN	42	39	39	42
	S.D.	5.8	9.0	10.0	7.3
	N	20	10	10	20
DAY 28	MEAN	35	31	38	34
	S.D.	5.6	6.3	6.7	8.1
	N	20	10	10	20
DAY 35	MEAN	11	--	--	15
	S.D.	5.8	--	--	4.3
	N	10	0	0	10
DAY 42	MEAN	27	--	--	27
	S.D.	5.5	--	--	2.6
	N	10	0	0	10
TOTAL GAIN	MEAN	195	--	--	200
	S.D.	15.4	--	--	32.9
	N	10	0	0	10

* P less than .05
** P less than .01
-- = Data Unavailable

Analysis of Variance using DUNNETT'S Procedure

TABLE 4 (cont.)

SUMMARY OF WEIGHT GAINS (Grams)

STUDY: 1209SN1 SEX: FEMALE

PERIOD	DOSE: (mg/kg) GROUP:	0 1-F	.0025 2-F	.005 3-F	.01 4-F
DAY 7	MEAN	15	14	14	17
	S.D.	6.7	5.4	4.3	4.6
	N	20	10	10	20
DAY 14	MEAN	17	17	19	17
	S.D.	4.6	5.6	4.3	3.7
	N	20	10	10	20
DAY 21	MEAN	16	14	14	14
	S.D.	4.3	3.6	3.9	2.7
	N	20	10	10	20
DAY 28	MEAN	12	10	13	10
	S.D.	4.5	3.2	3.9	3.2
	N	20	10	10	20
DAY 35	MEAN	5	--	--	4
	S.D.	4.4	--	--	5.3
	N	10	0	0	10
DAY 42	MEAN	9	--	--	8
	S.D.	3.7	--	--	4.8
	N	10	0	0	10
TOTAL GAIN	MEAN	66	--	--	76
	S.D.	8.4	--	--	13.5
	N	10	0	0	10

Analysis of Variance using DUNNETT'S Procedure

* P less than .05
** P less than .01
-- = Data Unavailable

TABLE 5

SUMMARY OF FOOD CONSUMPTION (Grams)

STUDY: 1209SN1 SEX: MALE

PERIOD	DOSE: (mg/kg) GROUP:	0 1-M	.0025 2-M	.005 3-M	.01 4-M
DAY 7	MEAN	166	166	171	173
	S.D.	21.0	9.2	13.3	10.6
	N	20	10	10	20
DAY 14	MEAN	167	157	163	164
	S.D.	16.0	12.3	14.3	15.9
	N	20	10	10	20
DAY 21	MEAN	177	160	171	172
	S.D.	15.7	17.3	17.4	16.0
	N	20	10	10	20
DAY 28	MEAN	176	161	177	175
	S.D.	16.7	16.3	16.6	18.5
	N	19	10	8	18
DAY 35	MEAN	179	-	-	178
	S.D.	14.3	-	-	20.8
	N	10	0	0	10
DAY 42	MEAN	201	-	-	197
	S.D.	15.2	-	-	18.5
	N	10	0	0	10

* P less than .05
** P less than .01
-- = Data Unavailable

Analysis of Variance using DUNNETT'S Procedure

TABLE 5 (cont.)

SUMMARY OF FOOD CONSUMPTION (Grams)

STUDY: 1209SN1 SEX: FEMALE

PERIOD	DOSE: (mg/kg) / GROUP:	0 1-F	.0025 2-F	.005 3-F	.01 4-F
DAY 7	MEAN	115	115	113	118
	S.D.	14.1	10.4	9.8	8.4
	N	20	10	10	20
DAY 14	MEAN	119	112	115	118
	S.D.	13.2	8.9	10.7	11.2
	N	20	10	10	20
DAY 21	MEAN	119	110	118	119
	S.D.	11.9	7.0	8.4	10.7
	N	20	9	10	19
DAY 28	MEAN	117	111	113	114
	S.D.	9.9	6.6	7.4	10.0
	N	20	9	10	19
DAY 35	MEAN	118	-	-	124
	S.D.	5.5	-	-	11.1
	N	10	0	0	10
DAY 42	MEAN	138	-	-	141
	S.D.	11.2	-	-	16.6
	N	10	0	0	10

* P less than .05
** P less than .01
-- = Data Unavailable

· Analysis of Variance using DUNNETT'S Procedure

TABLE 6

SUMMARY OF HEMATOLOGY DATA
PERIOD: Week 4

STUDY ID: 1209SN1 SEX: MALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(s): / UNITS:	WBC thsn/cmm	RBC mill/cmm	HGB g/dL	HCT %	MCV fl	MCH pg	MCHC %	PLT thsn/cmm	RETPC %
Group: 1-M : 0 ug/kg									
MEAN	17.4	7.80	16.1	46.9	60.1	20.6	34.3	1142	3.9
SD	4.51	0.379	0.57	2.14	1.48	0.61	0.84	89.0	0.81
N	20	20	20	20	20	20	20	20	20
Group: 2-M : 2.5 ug/kg									
MEAN	17.3	7.70	16.0	46.1	59.9	20.8	34.7	1140	3.3
SD	5.07	0.454	0.76	3.00	1.27	0.54	0.82	84.4	0.68
N	10	10	10	10	10	10	10	10	10
Group: 3-M : 5.0 ug/kg									
MEAN	16.9	7.76	16.0	46.9	60.5	20.6	34.0	1105	3.8
SD	4.46	0.408	0.43	1.68	1.89	0.94	1.22	91.8	0.53
N	10	10	10	10	10	10	10	10	10
Group: 4-M : 10.0 ug/kg									
MEAN	14.7	7.58	15.6	45.7	60.3	20.6	34.2	1089	3.6
SD	4.49	0.486	0.90	2.88	1.78	0.79	1.12	110.0	0.51
N	20	20	20	20	20	20	20	20	20

WBC corrected for NRBC > 0

TABLE 6 (cont.)

SUMMARY OF HEMATOLOGY DATA
PERIOD: Week 4

STUDY ID: 1209SN1 SEX: MALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(s): / UNITS:	RETABS thsn/cmm	NRBC #/100 WBC	MAT NEU thsn/cmm	LYMPH thsn/cmm	MONO thsn/cmm	EOSIN thsn/cmm	BASO thsn/cmm	IMM NEU thsn/cmm
Group: 1-M : 0 ug/kg								
MEAN	303.3	0.0	3.0	13.8	0.5	0.1	0.0	0.0
SD	63.64	0.00	0.94	3.80	0.18	0.12	0.00	0.00
N	20	20	20	20	20	20	20	20
Group: 2-M : 2.5 ug/kg								
MEAN	255.8	0.0	3.0	13.9	0.4	0.1	0.0	0.0
SD	51.75	0.00	0.88	4.43	0.23	0.12	0.00	0.00
N	10	10	10	10	10	10	10	10
Group: 3-M : 5.0 ug/kg								
MEAN	296.7	0.0	3.1	13.4	0.4	0.0	0.0	0.0
SD	34.12	0.00	1.00	3.65	0.18	0.06	0.00	0.00
N	10	10	10	10	10	10	10	10
Group: 4-M : 10.0 ug/kg								
MEAN	270.2	0.0	2.6	11.6	0.3	0.0	0.0	0.0
SD	45.91	0.00	0.88	3.64	0.22	0.08	0.00	0.00
N	20	20	20	20	20	20	20	20

WBC corrected for NRBC > 0

TABLE 6 (cont.)

SUMMARY OF HEMATOLOGY DATA
PERIOD: Week 4

STUDY ID: 1209SN1 SEX: MALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(s):	NRBC#	MAT NEU	LYMPH	MONO	EOSIN	BASO	IMM NEU
UNITS:	#/100 WBC	%	%	%	%	%	%
Group: 1-M : 0 ug/kg							
MEAN	0	18	79	3	1	0	0
SD	0.0	4.2	4.4	0.9	0.7	0.0	0.0
N	20	20	20	20	20	20	20
Group: 2-M : 2.5 ug/kg							
MEAN	0	17	80	3	0	0	0
SD	0.0	3.2	4.3	1.3	0.7	0.0	0.0
N	10	10	10	10	10	10	10
Group: 3-M : 5.0 ug/kg							
MEAN	0	19	79	3	0*	0	0
SD	0.0	3.4	4.1	1.1	0.3	0.0	0.0
N	10	10	10	10	10	10	10
Group: 4-M : 10.0 ug/kg							
MEAN	0	18	79	2	0	0	0
SD	0.0	2.4	2.7	1.4	0.6	0.0	0.0
N	20	20	20	20	20	20	20

*-Significant Difference from Control P < .05

TABLE 6 (cont.)

SUMMARY OF HEMATOLOGY DATA
PERIOD: Week 4

STUDY ID: 1209SN1 SEX: FEMALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(s): / UNITS:	WBC thsn/cmm	RBC mill/cmm	HGB g/dL	HCT %	MCV fl	MCH pg	MCHC %	PLT thsn/cmm	RETPC %
Group: 1-F : 0 ug/kg									
MEAN	13.2	7.83	15.9	48.1	61.5	20.3	33.1	1078	3.0
SD	1.78	0.318	0.40	1.15	1.69	0.79	0.69	97.1	0.62
N	20	20	20	20	20	20	20	20	20
Group: 2-F : 2.5 ug/kg									
MEAN	13.6	7.61	15.8	46.5	61.1	20.7	33.9**	1111	3.0
SD	2.81	0.332	0.62	1.70	1.79	0.80	0.63	122.5	0.58
N	10	10	10	10	10	10	10	10	10
Group: 3-F : 5.0 ug/kg									
MEAN	12.8	7.64	15.9	47.1	61.7	20.8	33.7*	1124	2.9
SD	3.18	0.288	0.51	1.69	1.04	0.57	0.92	116.3	0.50
N	10	10	10	10	10	10	10	10	10
Group: 4-F : 10.0 ug/kg									
MEAN	14.5	7.63	15.5	47.1	61.8	20.4	32.9	1087	3.0
SD	3.98	0.270	0.56	1.74	1.66	0.46	0.56	91.3	0.63
N	20	20	20	20	20	20	20	20	20

WBC corrected for NRBC > 0

**-Significant Difference from Control P < .01

*-Significant Difference from Control P < .05

TABLE 6 (cont.)

SUMMARY OF HEMATOLOGY DATA
PERIOD: Week 4

STUDY ID: 1209SN1 SEX: FEMALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(s): / UNITS:	RETABS thsn/cmm	NRBC #/100 WBC	MAT NEU thsn/cmm	LYMPH thsn/cmm	MONO thsn/cmm	EOSIN thsn/cmm	BASO thsn/cmm	IMM NEU thsn/cmm
Group: 1-F : 0 ug/kg								
MEAN	231.0	0.0	2.0	10.9	0.3	0.1	0.0	0.0
SD	48.88	0.00	0.60	1.59	0.14	0.12	0.00	0.00
N	20	20	20	20	20	20	20	20
Group: 2-F : 2.5 ug/kg								
MEAN	230.1	0.0	2.2	11.0	0.3	0.1	0.0	0.0
SD	47.35	0.00	0.58	2.18	0.22	0.11	0.00	0.00
N	10	10	10	10	10	10	10	10
Group: 3-F : 5.0 ug/kg								
MEAN	218.8	0.0	1.9	10.6	0.3	0.1	0.0	0.0
SD	41.53	0.00	0.61	2.70	0.11	0.10	0.00	0.00
N	10	10	10	10	10	10	10	10
Group: 4-F : 10.0 ug/kg								
MEAN	232.1	0.0	2.1	12.0	0.2	0.1	0.0	0.0
SD	48.29	0.00	0.56	3.59	0.17	0.12	0.00	0.00
N	20	20	20	20	20	20	20	20

WBC corrected for NRBC > 0

TABLE 6 (cont.)

SUMMARY OF HEMATOLOGY DATA
PERIOD: Week 4

STUDY ID: 1209SN1 SEX: FEMALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(s):	NRBC#	MAT NEU	LYMPH	MONO	EOSIN	BASO	IMM NEU
UNITS:	#/100 WBC	%	%	%	%	%	%
Group: 1-F : 0 ug/kg							
MEAN	0	15	82	2	1	0	0
SD	0.0	4.0	4.2	1.1	0.8	0.0	0.0
N	20	20	20	20	20	20	20
Group: 2-F : 2.5 ug/kg							
MEAN	0	16	81	2	1	0	0
SD	0.0	2.2	2.3	1.4	0.7	0.0	0.0
N	10	10	10	10	10	10	10
Group: 3-F : 5.0 ug/kg							
MEAN	0	15	83	2	0	0	0
SD	0.0	3.0	3.6	0.8	0.5	0.0	0.0
N	10	10	10	10	10	10	10
Group: 4-F : 10.0 ug/kg							
MEAN	0	15	83	2	1	0	0
SD	0.0	3.4	3.5	0.9	0.8	0.0	0.0
N	20	20	20	20	20	20	20

TABLE 7

SUMMARY OF HEMATOLOGY DATA
PERIOD: Recovery

STUDY ID: 1209SN1 SEX: MALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(s):	WBC	RBC	HGB	HCT	MCV	MCH	MCHC	PLT	RETPC
UNITS:	thsn/cmm	mill/cmm	g/dL	%	fl	pg	%	thsn/cmm	%
Group: 1-M : 0 ug/kg									
MEAN	15.7	8.14	16.3	46.8	57.5	20.0	34.8	1132	3.3
SD	2.65	0.278	0.33	1.21	1.31	0.76	0.99	97.7	0.53
N	10	10	10	10	10	10	10	10	10
Group: 4-M : 10.0 ug/kg									
MEAN	14.1	7.99	15.8	45.8	57.3	19.8	34.4	1111	3.5
SD	2.45	0.503	0.84	2.32	1.91	0.71	0.47	124.3	0.42
N	10	10	10	10	10	10	10	10	10

WBC corrected for NRBC > 0

TABLE 7 (cont.)

SUMMARY OF HEMATOLOGY DATA
PERIOD: Recovery

STUDY ID: 1209SN1 SEX: MALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(s): / UNITS:	RETABS thsn/cmm	NRBC #/100 WBC	MAT NEU thsn/cmm	LYMPH thsn/cmm	MONO thsn/cmm	EOSIN thsn/cmm	BASO thsn/cmm	IMM NEU thsn/cmm
Group: 1-M : 0 ug/kg								
MEAN	269.4	0.0	2.0	13.3	0.3	0.1	0.0	0.0
SD	44.57	0.00	0.38	2.52	0.13	0.11	0.00	0.00
N	10	10	10	10	10	10	10	10
Group: 4-M : 10.0 ug/kg								
MEAN	278.9	0.0	2.0	11.9	0.2	0.1	0.0	0.0
SD	37.53	0.00	0.51	2.17	0.20	0.08	0.00	0.00
N	10	10	10	10	10	10	10	10

WBC corrected for NRBC > 0

TABLE 7 (cont.)

SUMMARY OF HEMATOLOGY DATA
PERIOD: Recovery

STUDY ID: 1209SN1 SEX: MALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(s):	NRBC#	MAT NEU	LYMPH	MONO	EOSIN	BASO	IMM NEU
UNITS:	#/100 WBC	%	%	%	%	%	%
Group: 1-M : 0 ug/kg							
MEAN	0	13	84	2	1	0	0
SD	0.0	2.4	2.9	0.9	0.7	0.0	0.0
N	10	10	10	10	10	10	10
Group: 4-M : 10.0 ug/kg							
MEAN	0	14	84	2	1	0	0
SD	0.0	3.3	3.6	1.1	0.5	0.0	0.0
N	10	10	10	10	10	10	10

TABLE 7 (cont.)

SUMMARY OF HEMATOLOGY DATA
PERIOD: Recovery

STUDY ID: 1209SN1 SEX: FEMALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(s):	WBC	RBC	HGB	HCT	MCV	MCH	MCHC	PLT	RETPC
UNITS:	thsn/cmm	mill/cmm	g/dL	%	fl	pg	%	thsn/cmm	%
Group: 1-F : 0 ug/kg									
MEAN	10.9	7.79	15.7	46.6	59.8	20.2	33.8	1129	3.3
SD	2.47	0.284	0.55	1.60	1.89	0.79	0.65	114.6	0.52
N	10	10	10	10	10	10	10	10	10
Group: 4-F : 10.0 ug/kg									
MEAN	11.4	7.53	15.4	46.2	61.3*	20.5	33.4	1153	3.1
SD	2.63	0.266	0.52	1.82	1.08	0.70	0.97	107.6	0.48
N	10	10	10	10	10	10	10	10	10

WBC corrected for NRBC > 0

*-Significant Difference from Control P < .05

TABLE 7 (cont.)

SUMMARY OF HEMATOLOGY DATA
PERIOD: Recovery

STUDY ID: 1209SN1 SEX: FEMALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(s): / UNITS:	RETABS thsn/cmm	NRBC #/100 WBC	MAT NEU thsn/cmm	LYMPH thsn/cmm	MONO thsn/cmm	EOSIN thsn/cmm	BASO thsn/cmm	IMM NEU thsn/cmm
Group: 1-F : 0 ug/kg								
MEAN	254.6	0.0	1.5	9.1	0.3	0.1	0.0	0.0
SD	44.89	0.00	0.57	1.84	0.18	0.13	0.00	0.00
N	10	10	10	10	10	10	10	10
Group: 4-F : 10.0 ug/kg								
MEAN	233.9	0.0	1.7	9.5	0.2	0.1	0.0	0.0
SD	40.71	0.00	0.48	2.19	0.09	0.11	0.00	0.00
N	10	10	10	10	10	10	10	10

WBC corrected for NRBC > 0

TABLE 7 (cont.)

SUMMARY OF HEMATOLOGY DATA
PERIOD: Recovery

STUDY ID: 1209SN1 SEX: FEMALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(s):	NRBC#	MAT NEU	LYMPH	MONO	EOSIN	BASO	IMM NEU
UNITS:	#/100 WBC	%	%	%	%	%	%
Group: 1-F : 0 ug/kg							
MEAN	0	13	84	2	1	0	0
SD	0.0	2.4	3.4	1.4	1.1	0.0	0.0
N	10	10	10	10	10	10	10
Group: 4-F : 10.0 ug/kg							
MEAN	0	15	83	2	0	0	0
SD	0.0	2.2	2.8	0.7	0.8	0.0	0.0
N	10	10	10	10	10	10	10

TABLE 8

SUMMARY OF COAGULATION DATA
PERIOD: Week 4

STUDY ID: 1209SN1 SEX: MALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(s):	PT	APTT	FIBRINOGEN
UNITS:	sec	sec	mg/dL
Group: 1-M : 0 ug/kg			
MEAN	15.3	22.1	235
SD	0.72	2.13	14.8
N	10	10	10
Group: 2-M : 2.5 ug/kg			
MEAN	15.2	23.7	215**
SD	0.49	1.49	14.9
N	10	10	10
Group: 3-M : 5.0 ug/kg			
MEAN	15.1	22.1	222
SD	0.43	1.23	11.6
N	10	10	10
Group: 4-M : 10.0 ug/kg			
MEAN	14.8	22.5	215**
SD	0.42	1.64	9.5
N	10	10	10

**-Significant Difference from Control P < .01

TABLE 8 (cont.)

SUMMARY OF COAGULATION DATA
PERIOD: Week 4

STUDY ID: 1209SN1 SEX: FEMALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(s):	PT	APTT	FIBRINOGEN
UNITS:	sec	sec	mg/dL
Group: 1-F : 0 ug/kg			
MEAN	15.1	18.4	192
SD	0.46	1.27	17.4
N	10	10	10
Group: 2-F : 2.5 ug/kg			
MEAN	15.2	18.5	180
SD	0.24	1.05	10.9
N	10	10	10
Group: 3-F : 5.0 ug/kg			
MEAN	15.2	18.9	188
SD	0.46	1.46	16.7
N	10	10	10
Group: 4-F : 10.0 ug/kg			
MEAN	15.1	18.9	197
SD	0.43	1.19	13.8
N	10	10	10

TABLE 9

SUMMARY OF COAGULATION DATA
PERIOD: Recovery

STUDY ID: 1209SN1 SEX: MALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(s):	PT	APTT	FIBRINOGEN
UNITS:	sec	sec	mg/dL
Group: 1-M : 0 ug/kg			
MEAN	15.0	19.7	212
SD	0.75	2.12	14.8
N	10	10	10
Group: 4-M : 10.0 ug/kg			
MEAN	14.7	20.4	208
SD	0.28	2.32	18.7
N	10	10	10

TABLE 9 (cont.)

SUMMARY OF COAGULATION DATA
PERIOD: Recovery

STUDY ID: 1209SN1 SEX: FEMALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(s):	PT	APTT	FIBRINOGEN
UNITS:	sec	sec	mg/dL
Group: 1-F : 0 ug/kg			
MEAN	14.7	16.9	165
SD	0.41	1.62	8.6
N	10	10	10
Group: 4-F : 10.0 ug/kg			
MEAN	14.6	16.4	176
SD	0.58	2.05	13.3
N	10	10	10

TABLE 10

SUMMARY OF CLINICAL CHEMISTRY DATA
PERIOD: Week 4

STUDY ID: 1209SN1 SEX: MALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(s):	NA	K	CL	CA	PO4	CHOL	TG
UNITS:	mmol/L	mmol/L	mmol/L	mg/dL	mg/dL	mg/dL	mg/dL
Group: 1-M : 0 ug/kg							
MEAN	149	6.3	101	11.0	9.1	37	53
SD	1.2	0.58	2.0	0.46	0.79	7.0	25.2
N	20	20	20	20	20	20	20
Group: 2-M : 2.5 ug/kg							
MEAN	148	6.7	101	11.2	9.7	35	43
SD	1.5	0.88	2.4	0.35	1.05	4.9	16.4
N	10	10	10	10	10	10	10
Group: 3-M : 5.0 ug/kg							
MEAN	148	6.5	101	11.4	10.1	35	42
SD	1.4	0.65	1.9	0.35	0.87	6.1	19.7
N	10	10	10	10	10	10	10
Group: 4-M : 10.0 ug/kg							
MEAN	148	6.5	102	11.6**	10.7**	38	58
SD	2.4	0.69	2.5	0.73	1.59	5.1	24.6
N	20	20	20	20	20	20	20

**-Significant Difference from Control P < .01

TABLE 10 (cont.)

SUMMARY OF CLINICAL CHEMISTRY DATA
PERIOD: Week 4

STUDY ID: 1209SN1 SEX: MALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(s):	ALP	ALT	AST	GGT	LDH	TBIL
UNITS:	IU/L	IU/L	IU/L	IU/L	IU/L	mg/dL
Group: 1-M : 0 ug/kg						
MEAN	173	29	98	2	205	0.53
SD	38.2	4.9	18.0	1.6	143.8	0.077
N	20	20	20	20	20	20
Group: 2-M : 2.5 ug/kg						
MEAN	202	30	100	2	297	0.54
SD	51.5	3.9	15.7	1.1	264.7	0.074
N	10	10	10	10	10	10
Group: 3-M : 5.0 ug/kg						
MEAN	199	29	89	2	109	0.53
SD	86.6	4.5	8.3	1.0	33.4	0.064
N	10	10	10	10	10	10
Group: 4-M : 10.0 ug/kg						
MEAN	155	30	101	1	197	0.56
SD	33.6	8.6	31.7	1.1	122.0	0.108
N	20	20	20	20	20	20

TABLE 10 (cont.)

SUMMARY OF CLINICAL CHEMISTRY DATA
PERIOD: Week 4

STUDY ID: 1209SN1 SEX: MALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(s): / UNITS:	BUN mg/dL	CREA mg/dL	GLU mg/dL	TP g/dL	ALB g/dL	GLOB g/dL	A/G -
Group: 1-M : 0 ug/kg							
MEAN	13	0.6	119	6.6	4.6	2.0	2.4
SD	1.9	0.07	15.2	0.30	0.19	0.22	0.29
N	20	20	20	20	20	20	20
Group: 2-M : 2.5 ug/kg							
MEAN	13	0.6	124	6.6	4.5	2.0	2.3
SD	1.4	0.08	17.7	0.24	0.22	0.18	0.25
N	10	10	10	10	10	10	10
Group: 3-M : 5.0 ug/kg							
MEAN	13	0.6	117	6.4	4.5	1.9	2.4
SD	1.8	0.04	31.9	0.32	0.20	0.19	0.21
N	10	10	10	10	10	10	10
Group: 4-M : 10.0 ug/kg							
MEAN	14	0.6	124	6.6	4.5	2.0	2.2
SD	2.1	0.08	19.6	0.28	0.25	0.13	0.20
N	20	20	20	20	20	20	20

TABLE 10 (cont.)

SUMMARY OF CLINICAL CHEMISTRY DATA
PERIOD: Week 4

STUDY ID: 1209SN1 SEX: FEMALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(s):	NA	K	CL	CA	PO4	CHOL	TG
UNITS:	mmol/L	mmol/L	mmol/L	mg/dL	mg/dL	mg/dL	mg/dL
Group: 1-F : 0 ug/kg							
MEAN	146	5.5	101	11.2	7.1	51	35
SD	1.4	0.36	2.3	0.29	0.98	12.6	8.4
N	20	20	20	20	20	20	20
Group: 2-F : 2.5 ug/kg							
MEAN	146	5.7	102	11.3	7.7	48	37
SD	1.3	0.34	2.4	0.13	0.65	7.8	6.2
N	10	10	10	10	10	10	10
Group: 3-F : 5.0 ug/kg							
MEAN	147	5.5	101	11.6**	7.5	49	36
SD	1.2	0.36	2.4	0.31	0.54	9.3	8.5
N	10	10	10	10	10	10	10
Group: 4-F : 10.0 ug/kg							
MEAN	146	5.5	101	11.9**	8.2**	50	42
SD	1.9	0.34	1.6	0.35	0.54	10.5	13.4
N	20	20	20	20	20	20	20

**-Significant Difference from Control P < .01

TABLE 10 (cont.)

SUMMARY OF CLINICAL CHEMISTRY DATA
PERIOD: Week 4

STUDY ID: 1209SN1 SEX: FEMALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(s):	ALP	ALT	AST	GGT	LDH	TBIL
UNITS:	IU/L	IU/L	IU/L	IU/L	IU/L	mg/dL
Group: 1-F : 0 ug/kg						
MEAN	125	30	90	3	180	0.53
SD	28.5	6.7	11.1	1.2	141.6	0.091
N	20	20	20	20	20	20
Group: 2-F : 2.5 ug/kg						
MEAN	126	25	83	3	128	0.59
SD	23.5	5.8	9.6	1.3	56.8	0.103
N	10	10	10	10	10	10
Group: 3-F : 5.0 ug/kg						
MEAN	122	25	84	2	137	0.55
SD	26.9	4.1	11.0	0.6	119.3	0.056
N	10	10	10	10	10	10
Group: 4-F : 10.0 ug/kg						
MEAN	121	29	84	2	136	0.58
SD	42.5	9.0	12.4	0.9	88.1	0.104
N	20	20	20	20	20	20

TABLE 10 (cont.)

SUMMARY OF CLINICAL CHEMISTRY DATA
PERIOD: Week 4

STUDY ID: 1209SN1 SEX: FEMALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(s):	BUN	CREA	GLU	TP	ALB	GLOB	A/G
UNITS:	mg/dL	mg/dL	mg/dL	g/dL	g/dL	g/dL	-
Group: 1-F : 0 ug/kg							
MEAN	16	0.6	106	7.1	5.1	2.0	2.5
SD	1.7	0.07	12.5	0.38	0.32	0.21	0.33
N	20	20	20	20	20	20	20
Group: 2-F : 2.5 ug/kg							
MEAN	16	0.6	108	6.8	4.9	1.9	2.6
SD	1.1	0.09	12.6	0.29	0.16	0.20	0.26
N	10	10	10	10	10	10	10
Group: 3-F : 5.0 ug/kg							
MEAN	15	0.6	106	7.0	5.1	1.9	2.7
SD	1.4	0.08	6.3	0.24	0.22	0.18	0.30
N	10	10	10	10	10	10	10
Group: 4-F : 10.0 ug/kg							
MEAN	17	0.6	104	7.0	5.1	1.9	2.7
SD	2.3	0.05	6.2	0.47	0.48	0.21	0.42
N	20	20	20	20	20	20	20

TABLE 11

SUMMARY OF CLINICAL CHEMISTRY DATA
PERIOD: Recovery

STUDY ID: 1209SN1 SEX: MALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(s):	NA	K	CL	CA	PO4	CHOL	TG
UNITS:	mmol/L	mmol/L	mmol/L	mg/dL	mg/dL	mg/dL	mg/dL
Group: 1-M : 0 ug/kg							
MEAN	148	6.2	99	11.0	8.6	40	47
SD	1.3	0.42	1.8	0.39	0.92	9.2	17.6
N	10	10	10	10	10	10	10
Group: 4-M : 10.0 ug/kg							
MEAN	147	6.4	100	11.0	8.2	41	47
SD	1.5	0.77	1.4	0.25	0.92	5.3	21.2
N	10	10	10	10	10	10	10

TABLE 11 (cont.)

SUMMARY OF CLINICAL CHEMISTRY DATA
PERIOD: Recovery

STUDY ID: 1209SN1 SEX: MALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(s):	BUN	CREA	GLU	TP	ALB	GLOB	A/G
UNITS:	mg/dL	mg/dL	mg/dL	g/dL	g/dL	g/dL	-
Group: 1-M : 0 ug/kg							
MEAN	14	0.5	104	6.7	4.5	2.2	2.0
SD	2.0	0.08	10.7	0.26	0.18	0.22	0.25
N	10	10	10	10	10	10	10
Group: 4-M : 10.0 ug/kg							
MEAN	14	0.6*	109	6.5	4.3	2.2	2.0
SD	1.3	0.07	8.7	0.21	0.18	0.12	0.13
N	10	10	10	10	10	10	10

*-Significant Difference from Control P < .05

TABLE 11 (cont.)

SUMMARY OF CLINICAL CHEMISTRY DATA
PERIOD: Recovery

STUDY ID: 1209SN1 SEX: MALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(s):	ALP	ALT	AST	GGT	LDH	TBIL
UNITS:	IU/L	IU/L	IU/L	IU/L	IU/L	mg/dL
Group: 1-M : 0 ug/kg						
MEAN	135	28	89	2	194	0.56
SD	37.7	2.6	11.5	2.0	156.1	0.085
N	10	10	10	10	10	10
Group: 4-M : 10.0 ug/kg						
MEAN	127	27	79	2	84*	0.55
SD	19.4	5.4	10.6	1.3	37.2	0.089
N	10	10	10	10	10	10

*-Significant Difference from Control P < .05

TABLE 11 (cont.)

SUMMARY OF CLINICAL CHEMISTRY DATA
PERIOD: Recovery

STUDY ID: 1209SN1 SEX: FEMALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(s): / UNITS:	NA mmol/L	K mmol/L	CL mmol/L	CA mg/dL	PO4 mg/dL	CHOL mg/dL	TG mg/dL
Group: 1-F : 0 ug/kg							
MEAN	147	5.7	102	11.2	6.1	56	41
SD	1.2	0.25	1.4	0.32	0.97	18.3	10.6
N	10	10	10	10	10	10	10
Group: 4-F : 10.0 ug/kg							
MEAN	146	6.1*	102	11.6	6.6	57	49
SD	1.0	0.39	1.8	0.46	0.71	14.9	16.9
N	10	10	10	10	10	10	10

*-Significant Difference from Control P < .05

TABLE 11 (cont.)

SUMMARY OF CLINICAL CHEMISTRY DATA
PERIOD: Recovery

STUDY ID: 1209SN1 SEX: FEMALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(s):	ALP	ALT	AST	GGT	LDH	TBIL
UNITS:	IU/L	IU/L	IU/L	IU/L	IU/L	mg/dL
Group: 1-F : 0 ug/kg						
MEAN	81	28	89	2	148	0.54
SD	15.4	6.9	5.3	1.2	101.0	0.090
N	10	10	10	10	10	10
Group: 4-F : 10.0 ug/kg						
MEAN	86	32	93	3	163	0.52
SD	25.8	15.7	17.1	1.4	52.5	0.100
N	10	10	10	10	10	10

TABLE 11 (cont.)

SUMMARY OF CLINICAL CHEMISTRY DATA
PERIOD: Recovery

STUDY ID: 1209SN1 SEX: FEMALE

ANALYSIS OF VARIANCE FOLLOWED BY DUNNETT'S PROCEDURE

TEST(S):	BUN	CREA	GLU	TP	ALB	GLOB	A/G
UNITS:	mg/dL	mg/dL	mg/dL	g/dL	g/dL	g/dL	-
Group: 1-F : 0 ug/kg							
MEAN	18	0.6	105	7.5	5.3	2.2	2.5
SD	1.2	0.10	10.2	0.48	0.42	0.25	0.35
N	10	10	10	10	10	10	10
Group: 4-F : 10.0 ug/kg							
MEAN	19	0.6	108	7.5	5.4	2.1	2.6
SD	1.8	0.08	3.6	0.54	0.54	0.18	0.36
N	10	10	10	10	10	10	10

FOUR-WEEK ORAL (GAVAGE) TOXICITY STUDY OF 1α-HYDROXYVITAMIN D_5 IN RATS

TABLE 12

Mean Absolute Organ Weights (g)
Male Rats - Day 29

Treatment Group		Adrenals	Brain	Heart	Kidneys	Liver	Spleen	Testes	Thymus	Thyroid[b]
Vehicle Control	Mean	0.060	2.060	1.370	3.090	12.010	0.720	3.240	0.560	0.028
(0 µg/kg)	SD[a]	0.009	0.067	0.129	0.339	1.129	0.104	0.133	0.148	0.006
	N	10	10	10	10	10	10	10	10	10
Low	Mean	0.059	2.020	1.310	2.760	11.430	0.750	3.280	0.650	0.031
(2.5 µg/kg)	SD	0.008	0.068	0.111	0.179	1.375	0.136	0.194	0.165	0.005
	N	10	10	10	10	10	10	10	10	10
Mid	Mean	0.068	2.080	1.340	2.880	11.900	0.750	3.250	0.620	0.032
(5.0 µg/kg)	SD	0.013	0.082	0.127	0.253	1.621	0.095	0.207	0.114	0.004
	N	10	10	10	10	10	10	10	10	10
High	Mean	0.069	2.090	1.240	2.950	12.400	0.710	3.280	0.620	0.031
(10.0 µg/kg)	SD	0.011	0.113	0.101	0.203	1.249	0.113	0.281	0.168	0.003
	N	10	10	10	10	10	10	10	10	10

[a] standard deviation
[b] including parathyroids

TABLE 12 (cont.)

Mean Absolute Organ Weights (g)
Female Rats - Day 29

Treatment Group		Adrenals	Brain	Heart	Kidneys	Liver	Ovaries	Spleen	Thymus	Thyroid[b]
Vehicle Control	Mean	0.075	1.94	0.89	1.75	7.61	0.103	0.54	0.51	0.026
(0 µg/kg)	SD[a]	0.011	0.068	0.068	0.153	0.761	0.019	0.093	0.066	0.004
	N	10	10	10	10	10	10	10	10	10
Low	Mean	0.073	1.87	0.86	1.74	6.94	0.097	0.06	0.51	0.022*
(2.5 µg/kg)	SD	0.010	0.067	0.052	0.126	0.612	0.016	0.085	0.173	0.003
	N	10	10	10	10	10	10	10	10	10
Mid	Mean	0.071	1.89	0.87	1.73	7.35	0.102	0.51	0.40	0.022*
(5.0 µg/kg)	SD	0.011	0.079	0.078	0.107	0.893	0.015	0.083	0.063	0.003
	N	10	10	10	10	10	10	10	10	10
High	Mean	0.077	1.89	0.85	1.79	7.17	0.099	0.55	0.51	0.021*
(10.0 µg/kg)	SD	0.008	0.091	0.069	0.252	0.652	0.020	0.104	0.126	0.003
	N	10	10	10	10	10	10	10	10	10

* significantly different from vehicle control, $p \leq 0.05$
[a] standard deviation
[b] including parathyroids

TABLE 13

Mean Organ-to-Body Weight Ratios[a]
Male Rats - Day 29

Treatment Group		FBW[b]	Adrenals	Brain	Heart	Kidneys	Liver	Spleen	Testes	Thymus	Thyroid[d]
Vehicle Control	Mean	406	0.015	0.51	0.34	0.76	2.96	0.18	0.80	0.14	0.007
(0 µg/kg)	SD[c]	22.0	0.002	0.026	0.028	0.084	0.203	0.023	0.063	0.036	0.002
	N	10	10	10	10	10	10	10	10	10	10
Low	Mean	382	0.016	0.53	0.34	0.73	2.99	0.20	0.86	0.17	0.008
(2.5 µg/kg)	SD	28.2	0.003	0.041	0.018	0.048	0.202	0.032	0.080	0.033	0.001
	N	10	10	10	10	10	10	10	10	10	10
Mid	Mean	399	0.017	0.52	0.34	0.72	2.98	0.19	0.82	0.16	0.008
(5.0 µg/kg)	SD	33.7	0.003	0.043	0.021	0.049	0.249	0.016	0.060	0.022	0.001
	N	10	10	10	10	10	10	10	10	10	10
High	Mean	401	0.017	0.52	0.31*	0.74	3.09	0.18	0.82	0.16	0.008
(10.0 µg/kg)	SD	21.7	0.003	0.030	0.021	0.035	0.166	0.028	0.066	0.040	0.001
	N	10	10	10	10	10	10	10	10	10	10

[a] Organ-to-Body Weight Ratio = [Absolute Organ weight (g) ÷ Fasted Body Weight (g)] x 100
* significantly different from vehicle control, ≤0.05
[b] FBW = Fasted Body Weight (g)
[c] standard deviation
[d] including parathyroids

TABLE 13 (cont.)

Mean Organ-to-Body Weight Ratios[a]
Female Rats - Day 29

Treatment Group		FBW[b]	Adrenals	Brain	Heart	Kidneys	Liver	Ovaries	Spleen	Thymus	Thyroid[d]
Vehicle Control	Mean	246	0.030	0.79	0.36	0.71	3.09	0.042	0.22	0.21	0.011
(0 µg/kg)	SD[c]	10.5	0.004	0.035	0.024	0.066	0.226	0.007	0.038	0.032	0.002
	N	10	10	10	10	10	10	10	10	10	10
Low	Mean	229*	0.032	0.82	0.38	0.76	3.02	0.042	0.24	0.22	0.009
(2.5 µg/kg)	SD	11.3	0.005	0.051	0.015	0.041	0.153	0.008	0.223	0.076	0.001
	N	10	10	10	10	10	10	10	10	10	10
Mid	Mean	232*	0.031	0.82	0.38	0.75	3.18	0.044	0.22	0.17	0.010
(5.0 µg/kg)	SD	7.6	0.005	0.037	0.029	0.042	0.409	0.006	0.034	0.027	0.001
	N	10	10	10	10	10	10	10	10	10	10
High	Mean	233*	0.033	0.82	0.36	0.77	3.08	0.043	0.24	0.22	0.009
(10.0 µg/kg)	SD	16.8	0.004	0.042	0.026	0.077	0.172	0.008	0.035	0.055	0.001
	N	10	10	10	10	10	10	10	10	10	10

[a] Organ-to-Body Weight Ratio = [Absolute Organ weight (g) ÷ Fasted Body Weight (g)] x 100
* significantly different from vehicle control, $p \leq 0.05$
[b] FBW = Fasted Body Weight (g)
[c] standard deviation
[d] including parathyroids

FOUR-WEEK ORAL (GAVAGE) TOXICITY STUDY OF 1α-HYDROXYVITAMIN D_5 IN RATS

TABLE 14

Mean Absolute Organ Weights (g)
Male Rats - Recovery

Treatment Group		Adrenals	Brain	Heart	Kidneys	Liver	Spleen	Testes	Thymus	Thyroid[b]
Vehicle Control	Mean	0.065	2.11	1.41	3.07	12.09	0.74	3.48	0.57	0.028
(0 µg/kg)	SD[a]	0.011	0.094	0.081	0.236	0.838	0.068	0.172	0.101	0.006
	N	10	10	10	10	10	10	10	10	10
High	Mean	0.063	2.15	1.50	3.04	12.53	0.71	3.33	0.49	0.027
(10.0 µg/kg)	SD	0.009	0.082	0.189	0.460	1.946	0.079	0.160	0.106	0.006
	N	10	10	10	10	10	10	10	10	10

[a] standard deviation
[b] including parathyroids

TABLE 14 (cont.)

Mean Absolute Organ Weights (g)
Female Rats - Recovery

Treatment Group		Adrenals	Brain	Heart	Kidneys	Liver	Ovaries	Spleen	Thymus	Thyroid[b]
Vehicle Control	Mean	0.068	1.92	0.87	1.80	7.31	0.089	0.55	0.43	0.022
(0 µg/kg)	SD[a]	0.009	0.090	0.075	0.131	0.416	0.020	0.060	0.088	0.004
	N	10	10	10	10	10	10	10	10	10
High	Mean	0.078*	1.95	0.96	1.85	7.65	0.106	0.52	0.37	0.020
(10.0 µg/kg)	SD	0.007	0.063	0.094	0.214	0.894	0.019	0.088	0.073	0.004
	N	10	10	10	10	10	10	10	10	10

* significantly different from vehicle control, $p \leq 0.05$
[a] standard deviation
[b] including parathyroids

TABLE 15

Mean Organ-to-Body Weight Ratios[a]
Male Rats - Recovery

Treatment Group		FBW[b]	Adrenals	Brain	Heart	Kidneys	Liver	Spleen	Testes	Thymus	Thyroid[d]
Vehicle Control	Mean	430	0.015	0.49	0.33	0.72	2.81	0.17	0.81	0.13	0.006
(0 µg/kg)	SD[c]	21.6	0.003	0.039	0.026	0.059	0.093	0.018	0.050	0.025	0.001
	N	10	10	10	10	10	10	10	10	10	10
High	Mean	440	0.014	0.49	0.34	0.70	2.84	0.16	0.76	0.11*	0.006
(10.0 µg/kg)	SD	37.0	0.002	0.036	0.025	0.125	0.313	0.026	0.078	0.017	0.001
	N	10	10	10	10	10	10	10	10	10	10

[a] Organ-to-Body Weight Ratio = [Absolute Organ weight (g) ÷ Fasted Body Weight (g)] x 100
* significantly different from vehicle control, $p \leq 0.05$
[b] FBW = Fasted Body Weight (g)
[c] standard deviation
[d] including parathyroids

TABLE 15 (cont.)

Mean Organ-to-Body Weight Ratios[a]
Female Rats - Recovery

Treatment Group		FBW[b]	Adrenals	Brain	Heart	Kidneys	Liver	Ovaries	Spleen	Thymus	Thyroid[d]
Vehicle Control	Mean	245	0.028	0.78	0.36	0.74	2.99	0.036	0.23	0.18	0.009
(0 µg/kg)	SD[c]	13.8	0.003	0.050	0.024	0.048	0.218	0.008	0.020	0.034	0.001
	N	10	10	10	10	10	10	10	10	10	10
High	Mean	256	0.031	0.77	0.38	0.72	2.99	0.041	0.20*	0.15*	0.008
(10.0 µg/kg)	SD	19.2	0.003	0.066	0.039	0.079	0.272	0.007	0.029	0.029	0.002
	N	10	10	10	10	10	10	10	10	10	10

[a] Organ-to-Body Weight Ratio = [Absolute Organ weight (g) ÷ Fasted Body Weight (g)] x 100
* significantly different from vehicle control, $p \leq 0.05$
[b] FBW = Fasted Body Weight (g)
[c] standard deviation
[d] including parathyroids

TABLE 16

Summary of Incidence[a] of Necropsy Observations

Day 29

Group:	Control (0 µg/kg)		Low (2.5 µg/kg)		Mid (5.0 µg/kg)		High (10.0 µg/kg)	
Tissue - lesion	Males	Females	Males	Females	Males	Females	Males	Females
(N):	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)
No Gross Lesions	6	1	5	3	7	3	5	6
Adrenal								
focus	--[b]	1	--	--	--	--	--	--
nodule	--	--	--	1	--	1	--	--
Eye								
pigmentation, dark	--	--	1	--	--	--	--	--
Kidney								
pigmentation, mottled	1	--	--	--	--	--	1	--
focus	1	--	--	--	--	--	--	--
Liver								
mass	--	1	--	--	--	--	--	--
Lung								
focus	1	--	2	1	1	--	3	--
Lymph Node (bronchial)								
enlarged	--	--	--	--	--	--	--	1
Lymph Node (mandibular)								
enlarged	--	--	2	1	1	--	--	2
pigmentation, red	--	6	2	1	1	4	5	--
pigmentation, mottled	2	1	1	1	1	1	--	--
Lymph Node (pancreatic)								
pigmentation, red	1	--	--	--	--	--	--	--
Thymus								
pigmentation, mottled	1	1	--	1	--	--	--	--
pigmentation, red	--	--	--	--	--	1	--	--
Urinary Bladder								
pigmentation, red	--	--	1	--	--	--	--	--
Uterus								
dilatation	--	1	--	1	--	5	--	2

[a] Number of animals exhibiting the lesion
[b] -- = zero incidence

TABLE 17

Summary of Incidence[a] of Necropsy Observations

Recovery

Group:	Control (0 µg/kg)		High (10.0 µg/kg)	
Tissue -lesion	Males	Females	Males	Females
(N):	(10)	(10)	(10)	(10)
No Gross Lesions	7	6	7	9
Eye				
pigmentation, red	--[b]	--	--	1
pigmentation, opaque	--	1	--	--
Kidney				
pigmentation, mottled	--	--	1	--
Lung				
Focus, red	--	1	--	--
Lymph Node (mandibular)				
pigmentation, red	3	3	1	1
Thymus				
pigmentation, mottled	--	--	1	--
Uterus				
dilatation	--	--	--	1

[a] Number of animals exhibiting the lesion
[b] -- = zero incidence

DRAFT PATHOLOGY REPORT FOR FOUR-WEEK ORAL (GAVAGE) TOXICITY STUDY OF 1 α-HYDROXYVITAMIN D_5 IN RATS IITRI PROJECT NUMBER 1209, STUDY NUMBER 1

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JULY 31, 2000

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SECTION I

PATHOLOGY NARRATIVE

DRAFT PATHOLOGY REPORT

FOUR-WEEK ORAL (GAVAGE) TOXICITY STUDY OF 1 α-HYDROXYVITAMIN D_5 IN RATS

INTRODUCTION

This pathology report, submitted by Pathology Associates International (PAI) to IIT Research Institute (IITRI), represents the histopathology findings for the study designated as "Four-Week Oral (Gavage) Toxicity Study of 1 α-Hydroxyvitamin D_5 in Rats," IITRI Project Number 1209, Study Number 1.

The study was conducted to evaluate the toxicity of 1α-hydroxyvitamin D_5 when administered orally to rats for four weeks, and to determine the reversibility of any observed toxic effects.

EXPERIMENTAL DESIGN AND METHODS

Three groups (groups 2-4), each composed of 10 male and 10 female rats were given the test article once daily by oral gavage in 5 ml/kg of test article vehicle (corn oil) for a minimum of 28 consecutive days. The dose levels administered were 2.5, 5.0, and 10.0 μg/kg for animals in the low, mid, and high dose groups, respectively. Also, one group (group 1), composed of 10 male and 10 female rats was given the test article vehicle alone once daily by oral gavage for a minimum of 28 consecutive days. Groups 1 and 4 also contained 10 animals/sex that served as recovery animals. These 40 recovery animals received the same dosing that the other animals in their respective groups received and were held for two additional weeks without treatment prior to necropsy. The experimental design is summarized in Table I (Summary of Experimental Design).

Animals were anesthetized by sodium pentobarbital administration and sacrificed by exsanguination. All necropsies were performed according to IITRI Standard Operating Procedures and were conducted by PAI personnel. Tissues required by the protocol (see Table II, Protocol-Required Tissues) were examined and placed in 10% neutral buffered formalin. A bone marrow smear slide was collected from each animal, fixed in methanol and retained.

Tissues required for histopathologic evaluation in groups 1 and 4 of the main study were trimmed and processed, and slides were prepared in accordance with PAI Standard Operating Procedures. These tissues were evaluated by light microscopy and the results were tabulated. Kidney and sternum were trimmed and processed and slides were prepared in accordance with PAI Standard Operating Procedures for the main study animals in groups 2 and 3 and for the recovery animals in groups 1 and 4.

Treatment-related lesions are summarized in Table III, Summary of Treatment-Related Lesions. Microscopic findings for all groups are summarized in the Project Summary tables (Section II).

The mean group severity scores are found in the Severity Summary tables (Section III). Where applicable, all tissue changes received a severity grade based upon the following scale: 1 = minimal, 2 = mild, 3 = moderate, and 4 = marked. The mean group severity was determined by dividing the sum of the severity scores by the number of tissues examined in the group. Microscopic findings in the protocol-required tissues for individual animals are presented in the Tabulated Animal Data tables (Section IV). The correlation of the necropsy findings and histopathology findings are reported in the Correlation of Gross and Microscopic (Micro) Findings (Section V). The codes used as entries in these tables are explained in the Report Codes Table.

The portion of this study performed by PAI was conducted in compliance with the US Food and Drug Administration's Good Laboratory Practice (GLP) Regulations for Nonclinical Laboratory Studies, 21 CFR Part 58.

RESULTS AND DISCUSSION

The Results and Discussion section is divided into three parts: Necropsy Findings, Diagnostic Terms, and Histopathology Findings. The Necropsy Findings portion describes lesions seen at necropsy or trimming. The Diagnostic Terms portion lists and clarifies diagnostic terminology that may be unclear. Terms listed in the Diagnostic Terms portion of this section include, but are not limited to, those that are considered to be test article-related. The Histopathology Findings portion of this section reports the results and provides discussion of the histopathologic evaluation of the tissues.

Necropsy Findings

All gross lesions were interpreted as incidental findings. Gross observations are listed in the Correlation of Gross and Microscopic (Micro) Findings report in Section V. Microscopic findings were correlated with gross lesions when possible.

Diagnostic Terms

The morphologic characteristics of observations and lesions which require comment are presented in subsequent paragraphs to aid in the interpretation of the data.

Kidney

Mineralization in medulla or at the corticomedullary junction was characterized by the presence of small foci (5-20 microns diameter) of basophilic material in tubule lumens. Mineralization of pelvic epithelium was characterized by the presence of deeply basophilic granular material either in pelvic lining epithelial cells or in the lumen of the renal pelvis. Basophilic tubules occurred as small foci of basophilic stained renal tubule epithelial cells in the renal cortex. Chronic inflammation of renal cortex was characterized by focal infiltration of the interstitial tissue, usually around foci of basophilic staining tubules, with lymphocytes.

Tubule dilatation was characterized by dilatation of focal regions of distal convoluted tubules in the renal cortex.

Bone, sternum

Cartilage degeneration was characterized by the focal presence of lightly basophilic amorphous material in place of mineralized cartilage at the interface of the cartilage with trabecular bone.

The remainder of the diagnoses used in this study were considered to be self-explanatory and were not discussed in this section.

Histopathology Findings

The incidence and severity of treatment-related histopathology findings are summarized in Tables IIIA and IIIB, Summary of Treatment-Related Lesions. These findings are further discussed by organ in this section of the narrative report.

Terminal Sacrifice

Kidney

Mineralization in medulla was observed in the high dose males (7/10, SEV = 0.70), the mid dose males (2/10, SEV = 0.20), one control male (1/10, SEV = 0.10), the high dose females (7/10, SEV = 0.90). The mid dose females (10/10, SEV = 1.20), the low dose females (7/10, SEV = 0.70), and the control females (4/10, SEV = 0.40). The incidence of mineralization in medulla was interpreted as increased in the high dose males and in the high, mid, and low dose females when compared to the respective controls for each sex.

Mineralization of pelvic epithelium was observed in the high dose males (4/10, SEV = 0.40), the mid dose males (3/10, SEV = 0.30), the high dose females (7/10, SEV = 0.70), the mid dose females (7/10, SEV = 0.70), and the low dose females (4/10, SEV = 0.40). The incidence of pelvic epithelial mineralization was interpreted as increased in the high and mid dose males and in the high, mid, and low dose females when compared to the respective controls for each sex.

Mineralization at the corticomedullary junction was observed in the high dose females (4/10, SEV = 0.50), the mid dose females (3/10, SEV = 0.30), and the low dose females (2/10, SEV = 0.20). The incidence of corticomedullary junction mineralization was interpreted as increased in the high, mid, and low dose females when compared to the female controls.

Basophilic tubules were observed in the high dose males (7/10, SEV = 0.70), the mid dose males (6/10, SEV = 0.60), the low dose males (5/10, SEV = 0.50), the control males (6/10, SEV = 0.70), the high dose females (5/10, SEV = 0.50), the mid dose females (5/10, SEV = 0.50), the low dose females (4/10, SEV = 0.40), and the control females (1/10, SEV = 0.10). The incidence of basophilic tubules was interpreted as increased in the high, mid, and low dose females when compared to the female controls.

Dilatation of renal tubules was observed in the high dose males (5/10, SEV = 0.60), the mid dose males (4/10, SEV = 0.50), the low dose males (2/10, SEV = 0.20), the control males (2/10, SEV = 0.40), the high dose females (7/10, SEV = 0.70), the mid dose females (8/10, SEV = 0.90), the low dose females (5/10, SEV = 0.50), and the control females (1/10, SEV = 0.10). The incidence of renal tubule dilatation was interpreted as slightly increased in the high, mid, and low dose females when compared to the female controls.

Bone, sternum

Degeneration of cartilage was observed in the high dose males (8/10, SEV = 0.80), the mid dose males (10/10, SEV = 1.10), the low dose males (8/10, SEV = 0.80), the control males (5/10, SEV = 0.60), the high dose females (10/10, SEV = 1.00), the mid dose females (10/10, SEV = 1.10), the low dose females (9/10, SEV = 0.90), and the control females (5/10, SEV = 0.50). The incidence of cartilage degeneration was interpreted as slightly increased in all treated groups in both sexes.

Recovery Sacrifice

Kidney

Mineralization of pelvic epithelium was observed in the high dose males (3/10, SEV = 0.30), and the high dose females (6/10, SEV = 0.60), but was not observed in the controls of either sex. This indicates a persistent treatment effect on pelvic epithelium mineralization in both sexes.

The incidence of mineralization in medulla was similar for the high dose males (5/10, SEV = 0.50), the control males (4/10, SEV = 0.40), the high dose females (8/10, SEV = 0.80), and the control females (6/10, SEV = 0.60).

The incidence of corticomedullary mineralization was similar in the high dose females (4/10, SEV = 0.40) and the control females (4/10, SEV = 0.40).

Basophilic tubules were observed in the high dose males (7/10, SEV = 0.70), the control males (6/10, SEV = 0.60), the high dose females (6/10, SEV = 0.60), and the control females (1/10, SEV = 0.10). The incidence of basophilic tubules was interpreted as increased in the high dose females when compared to the control females.

Dilatation of renal tubules was observed in the high dose males (4/10, SEV = 0.40), the control males (2/10, SEV = 0.20), the high dose females (6/10, SEV = 0.60), and the control females (2/10, SEV = 0.20). The incidence of renal tubule dilatation was interpreted as increased in the high dose females.

Bone, sternum

The incidence of cartilage degeneration was similar in the high dose males (10/10, SEV = 1.10), the control males (10/10, SEV = 1.00), the high dose females (10/10, SEV = 1.10), and the control females (9/10, SEV = 1.10).

General Discussion

All of the lesions observed in kidney and bone are typically present in rodent studies. Also, the severity of the lesions present was within the range (minimal to mild) of those that are usually observed as an incidental finding. Only the incidence of the lesions appears to be influenced by test article treatment. Mineralization of pelvic epithelium was the most treatment-specific finding, being present only in treated animals in this study. However, mineralization of pelvic epithelium does often occur in studies involving older rodents.

A dose-response relationship was apparent for mineralization in pelvic epithelium in males and to a lesser extent in females. A dose-response relationship was also apparent for mineralization of medulla in males. All other treatment-related findings (cartilage degeneration in males and females; mineralization in renal medulla in females, corticomedullary mineralization, basophilic renal tubules, and dilatation of renal tubules) were increased to a similar degree in the low, mid, and high dose animals.

CONCLUSIONS

Under the conditions of this study, daily administration of 1 α-hydroxyvitamin D_5 by oral gavage to rats for a minimum of 28 days at a dose of 10 μg/kg resulted in a dose-dependent increase in mineralization in renal medulla of males. Similar administration of 1 α-hydroxyvitamin D_5 at a dose of 5 or 10 μg/kg resulted in a dose-dependent increase in the incidence of mineralization of renal pelvic epithelium in males and females. Similar administration of 1 α-hydroxyvitamin D_5 at a dose of 2.5 μg/kg resulted in a dose-dependent increase in the incidence of mineralization of renal pelvic epithelium in females. The incidence of several lesions (cartilage degeneration in sternum from males and females; renal medulla mineralization in females; renal corticomedullary mineralization in females, basophilic tubules in females; and renal tubule dilatation in females) was also increased in a dose-independent manner in animals given 2.5, 5, or 10 μg/kg.

After a recovery period of approximately 2 weeks, the incidence of mineralization of renal pelvic epithelium was still slightly increased in males and females. The incidence of basophilic renal tubules and renal tubule dilatation was also still increased in recovery females.

A no-effect level was not established by this study. However, the toxicological significance of lesions occurring at all dose levels was considered to be minimal because of the low severity (minimal or mild) of the lesions and the occurrence of these lesions as incidental findings in most rodent studies.

_____ _____

Robert L. Morrissey, DVM, Ph.D. Date
Diplomate, ACVP

TABLE I

SUMMARY OF EXPERIMENTAL DESIGN

Group Number	Dose Group	Dose Level (µg/kg)	Number of Males	Number of Females
1	Control	0	20*	20*
2	Low	2.5	10	10
3	Mid	5.0	10	10
4	High	10.0	20*	20*

* Includes 10 animals/sex for recovery.

TABLE II

PROTOCOL-REQUIRED TISSUES

Adrenal glands
Aorta
Brain
Cecum
Colon
Duodenum
Epididymis
Esophagus
Eyes with optic nerve
Femur with marrow
Harderian gland
Heart
Ileum
Jejunum
Kidneys
Lacrimal glands
Liver
Lung with bronchi
Lymph node (mandibular)
Ovaries
Pancreas
Pituitary gland
Prostate with seminal vesicles
Rectum
Salivary gland (mandibular)
Sciatic nerve
Skeletal muscle
Skin with mammary gland
Spinal cord (cervical, thoracic, and lumbar)
Spleen
Sternum with bone marrow
Stomach
Testes
Thymus
Thyroid gland with parathyroids
Tongue
Trachea
Urinary bladder
Uterus
Vagina
Gross lesions
Bone marrow smear

TABLE IIIA (Terminal Sacrifice)
SUMMARY OF TREATMENT-RELATED LESIONS

		Dose (ug/kg)			
ORGAN - lesion		0	2.5	5.0	10.0
KIDNEY					
- Mineralization, medulla	M	1/10 (0.10)*	0/10	2/10 (0.20)	7/10 (0.70)
	F	4/10 (0.40)	7/10 (0.70)	10/10 (1.20)	7/10 (0.90)
- Mineralization, pelvis epithelium	M	0/10	0/10	3/10 (0.30)	4/10 (0.40)
	F	0/10	4/10 (0.40)	7/10 (0.70)	7/10 (0.70)
- Basophilic tubules	M	6/10 (0.70)	5/10 (0.50)	6/10 (0.60)	7/10 (0.70)
	F	1/10 (0.10)	4/10 (0.40)	5/10 (0.50)	5/10 (0.50)
- Dilatation, tubule	M	2/10 (0.40)	2/10 (0.20)	4/10 (0.50)	5/10 (0.60)
	F	1/10 (0.10)	5/10 (0.50)	8/10 (0.90)	7/10 (0.70)
- Mineralization, corticomedullary	M	0/10	0/10	0/10	0/10
	F	0/10	2/10 (0.20)	3/10 (0.30)	4/10 (0.50)
BONE, STERNUM					
- Degeneration, cartilage	M	5/10 (0.60)	8/10 (0.80)	10/10 (1.10)	8/10 (0.80)
	F	5/10 (0.50)	9/10 (0.90)	10/10 (1.10)	10/10 (1.00)

* Incidence (mean group severity score)

TABLE IIIB (Recovery Sacrifice)
SUMMARY OF TREATMENT-RELATED LESIONS

		Dose (ug/kg)	
ORGAN - lesion		0	10.0
KIDNEY			
- Mineralization, medulla	M	4/10 (0.40)*	5/10 (0.50)
	F	6/10 (0.60)	8/10 (0.80)
- Mineralization, pelvis epithelium	M	0/10	3/10 (0.30)
	F	0/10	6/10 (0.60)
- Basophilic tubules	M	6/10 (0.60)	7/10 (0.70)
	F	1/10 (0.10)	6/10 (0.60)
- Dilatation, tubule	M	2/10 (0.20)	4/10 (0.40)
	F	2/10 (0.20)	6/10 (0.60)
- Mineralization, corticomedullary	M	0/10	0/10
	F	4/10 (0.40)	4/10 (0.40)
BONE, STERNUM			
	M	10/10 (1.00)	10/10 (1.10)
	F	9/10 (1.10)	10/10 (1.10)

* Incidence (mean group severity score)

APPENDIX

QUALITY ASSURANCE STATEMENT

This histopathology project was inspected and audited by the PAI Quality Assurance Unit (QAU) as required by the Good Laboratory Practice (GLP) standards promulgated by the Food and Drug Administration. The pathology narrative report is an accurate reflection of the recorded data. The following table is a record of the inspections/audits performed and reported by the QAU:

Date of Inspection	Phase Inspected	Date Findings Reported to Management and Study Pathologist
05/12/00	Processing and Embedding	05/12/00
06/09/00	Individual Animal Data	06/09/00
06/09/00	Draft Pathology Report	06/09/00
07/31/00	Second Draft Pathology Report	07/31/00

Andrea Marshall

Andrea Marshall
Quality Assurance Unit
PAI Illinois Division

07/31/00

Date

Four-Week Oral (Gavage) Toxicity Study of 1α-Hydroxyvitamin D_5 in Rats
IITRI Project Number 1209, Study Number 1

Manuscript, European Journal of Cancer
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Induction of differentiation by 1α-hydroxyvitamin D_5 in T47D human breast cancer cells and its interaction with vitamin D receptors

G. Lazzaro [a], A. Agadir [b], W. Qing [a], M. Poria [a], R.R. Mehta [a], R.M. Moriarty [a], T.K. Das Gupta [a], X.-K. Zhang [b], R.G. Mehta [a,*]

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Induction of differentiation by 1α-hydroxyvitamin D_5 in T47D human breast cancer cells and its interaction with vitamin D receptors

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Abstract

The role of the active metabolite of vitamin D, 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), in cell differentiation is well established. However, its use as a differentiating agent in a clinical setting is precluded due to its hypercalcaemic activity. Recently, we synthesised a relatively non-calcaemic analogue of vitamin D_5, 1α-hydroxyvitamin D_5 ($1\alpha(OH)D_5$), which inhibited the development of carcinogen-induced mammary lesions in culture and suppressed the incidence of chemically induced mammary carcinogmas in rats. In the present study, we determined the differentiating effects of 1α-$(OH)D_5$ in T47D human breast cancer cells and compared its effects with $1,25(OH)_2D_3$. Cells incubated with either 10 or 100 nM of the analogues inhibited cell proliferation in a dose-dependent manner, as measured by the dimethylthiazolyl-2,5-diphenyltetrazolium bromide (MTT) assay. Similar growth-inhibitory effects were also observed for $MCF10_{neo}$ cells. Both vitamin D analogues induced cell differentiation, as determined by induction of casein expression and lipid production. However, $MCF10_{neo}$ cells failed to respond to either vitamin D analogue and did not undergo cell differentiation. Since the cell differentiating effect of vitamin D is considered to be mediated via the vitamin D receptor (VDR), we examined the induction of VDR using reverse transcriptase–polymerase chain reaction (RT-PCR) in both cells. The results showed that, in T47D cells, both $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ induced VDR in a dose-dependent manner. Moreover, both analogues of vitamin D upregulated the expression of vitamin D response element-chloramphenicol acetyl transferase (VDRE-CAT). These results collectively indicate that 1α-$(OH)D_5$ may mediate its cell-differentiating action via VDR in a manner similar to that of $1,25(OH)_2D_3$.

Keywords: Vitamin D; Breast cancer cells; Differentiation; T47D; MCF10

1. Introduction

The research on vitamin D_3 and related compounds is currently at its apex. A vast amount of evidence has been collected, implicating the essential involvement of vitamin D metabolites in several cellular processes. The active metabolite 1,25 dihydroxy vitamin D_3 ($1,25(OH)_2D_3$) and related compounds suppress the development and progression of breast cancer and other carcinomas *in vivo* [1,2], inhibit the metastatic spread of tumour cells [3–5], and promote differentiation of breast cancer cells [6–8]. However, the calcaemic side-effects of $1,25(OH)_2D_3$ have prevented its application as a pharmaceutical agent. In recent years, considerable attention has been given to the development of vitamin D_3 analogues capable of inducing cell differentiation without systemic hypercalcaemia [8–10]. Many structural modifications are known to enhance several-fold the differentiating potency of vitamin D_3 analogues in normal (usually keratinocyte) or malignant (usually leukaemia) cell lines. Little attempt, however, has been made to evaluate vitamin D analogues of other series such as vitamin D_2, D_4, D_5 and D_6. This structural classification is based on the differences encountered in the side chain. Earlier studies reported that vitamin D_5 was the least toxic of vitamins D_2 through to D_6 [11].

During the past 2 years, we have been studying the role of 1α-hydroxyvitamin D_5 ($1\alpha(OH)D_5$), an analogue of vitamin D_5 (24-ethyl-vitamin D_3), on breast cancer cell differentiation. We have characterised its calcaemic

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activity in vitamin D-deficient Sprague–Dawley rats [12]. The analogue $1\alpha(OH)D_5$ was synthesised from sitosterol acetate and was found to be less calcaemic than vitamin D_3. It was observed that $1\alpha(OH)D_5$ was effective against the development of carcinogen-induced mammary lesions in mouse mammary gland organ cultures [12]. In a more recent study, we observed that $1\alpha(OH)D_5$ inhibited incidence and tumour multiplicity of N-methyl-N-nitrosourea-induced mammary adenocarcinoma in rats (data not shown). These results clearly demonstrate that this vitamin D analogue might be a good candidate in the prevention of mammary carcinogenesis. In the present study, we evaluated the effects of $1\alpha(OH)D_5$ on cell differentiation and proliferation in oestrogen receptor (ER)-positive T47D breast cancer cells and compared the effects of the D_5 analogue with the active metabolite of vitamin D_3, $1,25(OH)_2D_3$. Moreover, we compared the effects of vitamin D analogues between ER+ T47D cells and ER- $MCF10_{neo}$ cells. Both cell lines are negative for functional p53 [13].

It is well known that the nuclear activity of vitamin D_3 is based on the interaction of the vitamin D active metabolite, $1,25(OH)_2D_3$, with the vitamin D receptor (VDR) [14]. The VDR is a nuclear receptor that belongs to the superfamily of ligand-dependent transcription factors and is expressed in all the vitamin D target tissues. VDR mediates its action by conjugating with the Retinoid X Receptor (RXR) [15–17]. The VDR-RXR dimer, once formed, is capable of recognising the vitamin D response element (VDRE) in the promoter region of the gene. The VDRE is composed of direct repeats of 6 DNA bases separated by 3-base intervening sequences [18]. Vitamin D appears to play an important role in stabilising and transactivating the VDR/RXR–VDRE complex [19,20]. Its interaction with VDR, therefore, represents the central step in the transmission of a signal to the transcription machinery, resulting in activation or suppression of transcription of genes leading ultimately to differentiation. We recently showed that the normal human breast epithelial cells lacking functional VDR do not respond to vitamin D to induce cell differentiation. However, transient transfection of VDR in these HBL-100 cells resulted in increased association of VDR-VDRE, as measured by the CAT reporter assay [21]. In the present study, we compared the effects of $1\alpha(OH)D_5$ and $1,25(OH)_2D_3$ on the transactivation of VDR–VDRE in T47D cells.

2. Materials and methods

2.1. Cells

The breast epithelial cell line, $MCF10_{neo}$, and human breast cancer cell line, T47D, were obtained from the American Type Culture Collection (Rockville, MD, USA). The $MCF10_{neo}$ cells were maintained in minimum essential medium with Earl's salts (MEME) medium supplemented with 10% fetal bovine serum (FBS), whereas T47D cells were maintained in RPMI supplemented with 0.2 I.U. bovine insulin/ml and 10% FBS. The monkey renal cancer CV-1 cells were maintained in MEME with 10% FBS supplement.

2.2. MTT assay

The cells were seeded in a 96-well/plate at a density of 500 cells/well in 100 μl/well of cell culture medium supplemented with 10% steroid-stripped serum. 24 h after seeding, the cells were incubated with 10 and 100 nM concentrations of $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$, respectively. The medium was changed every 3 days. After 7 days, the cultures were used for the dimethylthiazolyl-2,5-diphenyltetrazolium Bromide (MTT) assay. MTT (5 mg/ml in phosphate buffered serum (PBS)) was added to the wells (15 μl/well) and incubated at 37°C for 2 h. The stop solution (20% sodium dodecyl sulphate (SDS) in 50% N,N-dimethylformamide) was then added (100 μl/well) and incubated for an additional 2 h. The plates were scanned at 590 nm OD, and the results for each treatment group were averaged.

2.3. Immunohistochemistry

$MCF10_{neo}$ and T47D cells were grown to subconfluence on coverslips for 7 days, washed in PBS and fixed in 10% formalin for 5 min. The fixed cells were further incubated in cold methanol for 3 min and acetone for 2 min. After blocking the cells with a protein block/normal goat serum (BioGenex, San Ramos, CA, USA), they were incubated with casein antibody (100 μg/ml) (Accurate Chemical and Scientific Corp. Westbury, NY, USA) for 2 h. The cells were then incubated with secondary anti-mouse biotinylated antibody for 30 min, followed by streptavidin–peroxidase complex and 3,3′-diaminobenzidine (DAB) solution as chromogen. Appropriate controls were performed to rule out non-specific staining with secondary antibody.

2.4. Lipid assay

$MCF10_{neo}$ and T47D cells were grown to subconfluence on coverslips for 7 days, washed in PBS, and fixed by incubating in cold methanol for 3 min and propylene glycol for 2 min. The cells were, at this point, stained with Oil Red O' for 30 min and rinsed in isopropyl alcohol then de-ionised water. Haematoxylene staining for 30 s and Scott solution rinse completed the assay.

2.5. RNA isolation and RT-PCR

The cells were incubated with 1, 10 or 100 nM $1,25(OH)_2D_3$ or $1\alpha(OH)D_5$ for 3 days. The medium

from the tissue culture flasks was removed and the cells were treated with RNA-Zol B (Tel-Test Inc., Friendswood, TX, USA). RNA were isolated according to the manufacturer's instruction and quantified spectrophotometrically. Reverse transcription (RT) and PCR were carried out using Advantage RT for PCR and Advantage cDNA PCR kit (Clontech Inc., Palo Alto, CA, USA). Primer sequences for VDR were selected and custom-synthesised by Oligos Etc. The sense primer was 5′-GGA GTT GCT GTT TGT TTG AC, and the antisense primer was 5′-CTT CTG TGA GGC TGT TTT TG. The primer for the housekeeping gene *G3PDH* was purchased from ClonTech. The touchdown PCR procedure was employed with minor modifications [22]. The first strand cDNA was heated at 94°C for 1 min followed by denaturation at 94°C for 45 sec, annealing at 68–66–64–62–60°C for 45 sec each time and extension at 72°C for 2 min, for 26 cycles. The final cycle was followed by a 7-min extension step at 72°C to ensure that the amplified DNA was double stranded. The absence of contaminant was routinely checked by RT-PCR assays of negative control samples (sterile buffer, provided in the kit). The PCR products were separated on 1.5% agarose gel at 64 volts for 3 h, stained with ethidium bromide and visualised by ultraviolet (UV)-transillumination.

2.6. Transient transfection

The reporter construct VDRE-tk-CAT was prepared by inserting a copy of VDRE into the *Bam*HI site of the $pBLCAT_2$ as previously described [23]. For transfection, 1×10^5 CV-1 cells were plated in 24-well plates. Transfections were carried out using the calcium phosphate precipitation procedure. Briefly, 100 ng of VDRE-tk-CAT reporter plasmid, 250 ng of β-galactosidase (β-gal) expression vector, and 500 ng of VDR expression vectors were mixed with carrier DNA (pBluescript) to 1 μg of total DNA per well. The CAT activity was normalised for transfection efficiency by the corresponding β-gal activity.

3. Results

3.1. Effect of $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ on cell proliferation

The breast epithelial cells $MCF10_{neo}$ and breast cancer cells T47D were incubated with the vitamin D analogues for 7 days in culture. After this, the effects of vitamin D analogues were evaluated by the MTT assay. The results indicated a 31% and 50% growth inhibition for $MCF10_{neo}$ at 10 and 100 nM of $1,25(OH)_2D_3$ concentrations, respectively, as compared with 50% and 72% inhibition with $1\alpha(OH)D_5$ at 10 and 100 nM, respectively (Fig. 1a). The ER-positive, T47D cells showed a 29% and 52.5% growth inhibition after being exposed for 7 days to $1,25(OH)_2D_3$ at 10 and 100 nM, respectively. Unlike $MCF10_{neo}$ cells, T47D cells did not exhibit increased growth suppression when exposed to $1\alpha(OH)D_5$. Both analogues suppressed growth of T47D cells by approximately 30% and 50% at low and high concentrations, respectively (Fig. 1b). These results suggest that both $1\alpha(OH)D_5$ and $1,25(OH)_2D_3$ are comparable in producing antiproliferative effects in breast cancer cells.

3.2. Induction of differentiation of breast cancer cell lines

Since one of the major recognised functions of vitamin D is induction of cell differentiation, we evaluated the effects of both analogues on the induction of differentiation in both cell lines. As markers of cell differentiation, we used casein and lipid. Casein expression was measured by immunocytochemistry using casein antibodies. Results showed that, for T47D cells, casein was expressed in less than 10% of the control cells.

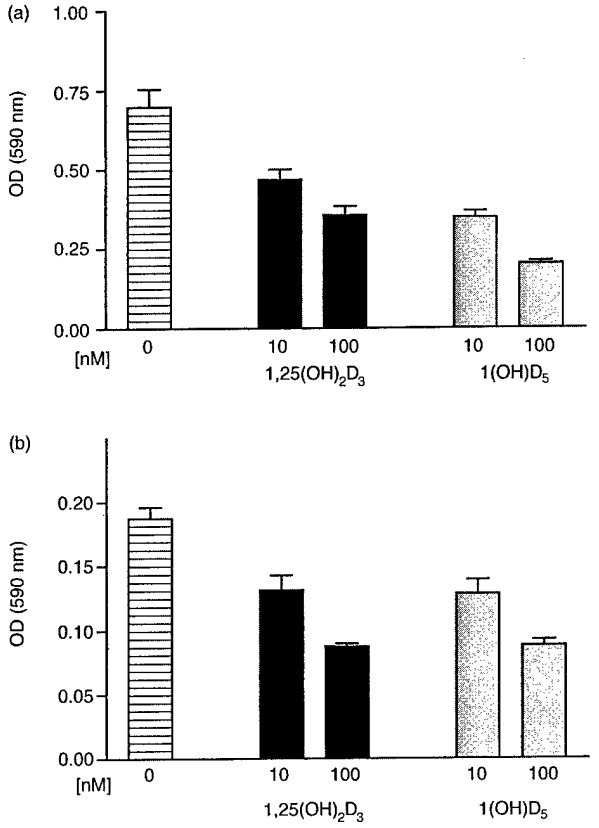

Fig. 1. Effects of $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ on the proliferation of $MCF10_{neo}$ cells and T47D cells. The MTT assay was carried out using duplicate cultures and the experiments were repeated three times. The error bars represent the standard deviation. (a) $MCF10_{neo}$ cells; (b) T47D cells.

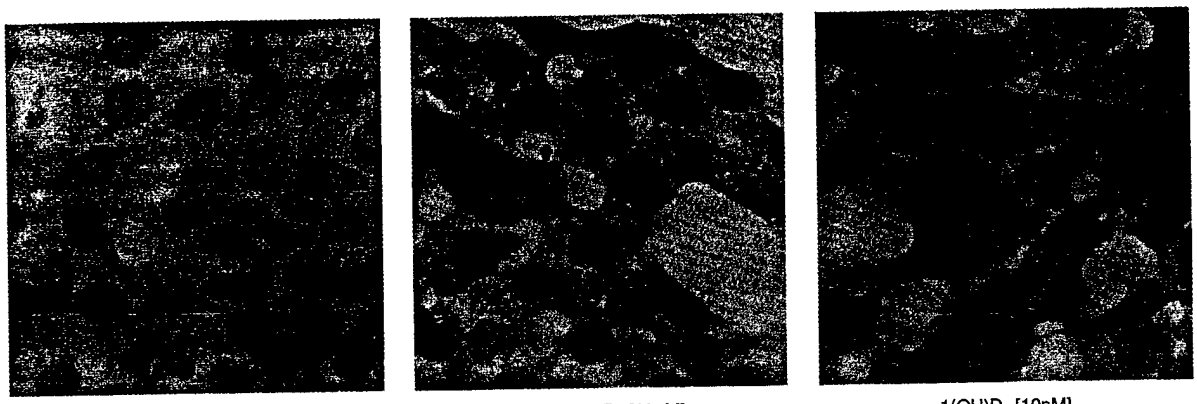

Fig. 2. Effect of vitamin D analogues on casein expression in T47D cells. Immunohistochemical staining for casein expression was carried out as previously described in the presence or absence of the vitamin D analogues.

After 7 days treatment with $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$, the intensity and number of cells expressing casein increased to approximately 70 and 85% at 10 and 100 nM concentrations, respectively. No difference was noticed between the effects of D_3 or D_5 analogues (Fig. 2 and data not shown). Similarly, there was a dramatic increase in the expression of lipid production in T47D cells after 7 days of treatment with $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ (Fig. 3). These results indicated that both vitamin D analogues induce cell differentiation in T47D cells. In contrast, the $MCF10_{neo}$ cells, tested for the same markers of differentiation, did not show any presence or induction of either casein or lipids in the control cells or in cells exposed to vitamin D_3 or D_5 (data not shown).

3.3. Transactivation of VDRE

The VDRE transactivation activity of the vitamin D analogues was determined using the *CAT* reporter gene containing VDRE (VDRE-tk-CAT). In order to compare the activity of $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ for transactivating the *VDRE* reporter gene, we selected monkey renal cancer cells (CV-1). These cells lack a functional VDR, so one can evaluate the binding activity of vitamin D analogues only in the transiently transfected VDR. The active metabolite of vitamin D, $1,25(OH)_2D_3$, should not show any increase in CAT activity if the cells are transfected only with VDRE-tk-CAT. As shown in Fig. 4, neither vitamin D_3 nor vitamin D_5 analogues could induce CAT activity, indicating a lack of endogenous VDR in these cells. However, when 500 ng VDR (Fig. 4b) was co-transfected with VDRE and the cells were incubated with 10 or 100 nM of $1,25(OH)_2D_3$ or $1\alpha(OH)D_5$, there was enhanced expression of the *CAT* reporter gene. These results clearly indicate that both analogues of vitamin D can bind to the VDR and the complex can bind to the VDRE to initiate signal transduction. However, the extent of VDRE-reporter transactivation was 7- to 8-fold greater when the transfected cells were incubated with $1,25(OH)_2D_3$ at 10 nM and nearly 2-fold greater at 100 nM, respectively, compared with $1\alpha(OH)D_5$ at the same concentrations. This is consistent with the observed

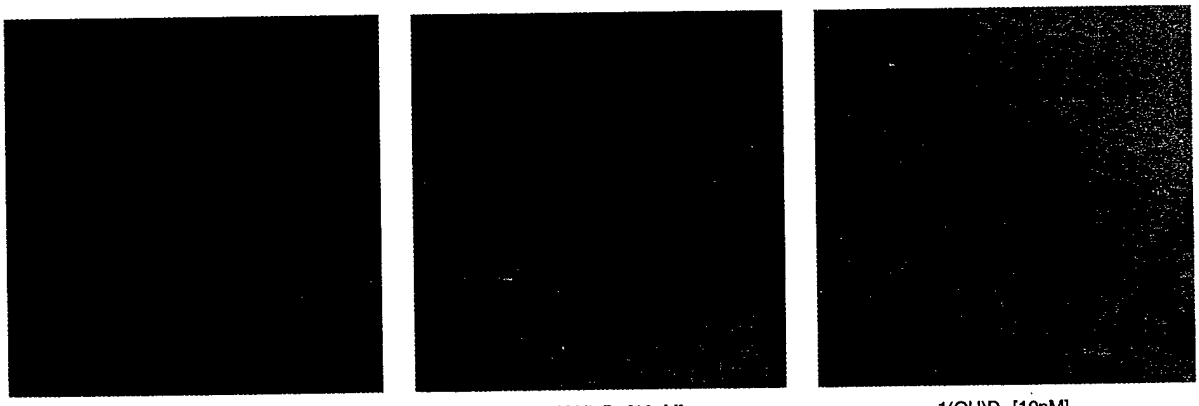

Fig. 3. Effects of $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ on lipid expression in T47D cells. A lipid assay was carried out as previously described in the presence or absence of vitamin D analogues.

finding that a log molar higher concentration of $1(OH)D_5$ is needed to obtain an equivalent response to that observed with $1,25(OH)_2D_3$.

3.4. Induction of VDR mRNA as determined by RT-PCR

Experiments were carried out to determine if VDR mRNA is induced by the vitamin D analogues in T47D and $MCF10_{neo}$ cells. Total RNA from the cells was isolated and reverse-transcribed. The cDNA was amplified using Taq polymerase and separated on 1.5% agarose gel. As shown in Fig. 5, the housekeeping gene *G3PDH* (C) was identical for all the cDNAs, indicating an equal loading of the gels. The VDR separated as a 420 bp fragment on the gel. As shown in Fig. 5(a), in T47D cells, there was a basal level of expression of VDR; however, incubation of cells for 3 days with either 10 or 100 nM of $1,25(OH)_2D_3$ increased the VDR expression in a dose-related manner. Similar results were also obtained with $1\alpha(OH)D_5$, as shown in Fig. 5(a). In contrast, $MCF10_{neo}$ cells expressed the basal level of VDR in the cells; but, there was no induction of VDR message by the vitamin D analogues (Fig. 5b). These results indicate that the lack of induction of differentiation by vitamin D in $MCF10_{neo}$ cells may be related to a lack of induction of VDR in these cells by vitamin D analogues.

4. Discussion

The effects of vitamin D analogues as differentiating agents and inhibitors of cell proliferation for breast cancer cells have been reported [1,7]. It is generally believed that the cells expressing VDR often respond to vitamin D analogues, whereas cells such as MDA-MB-231, which are ER- and express low or non-detectable

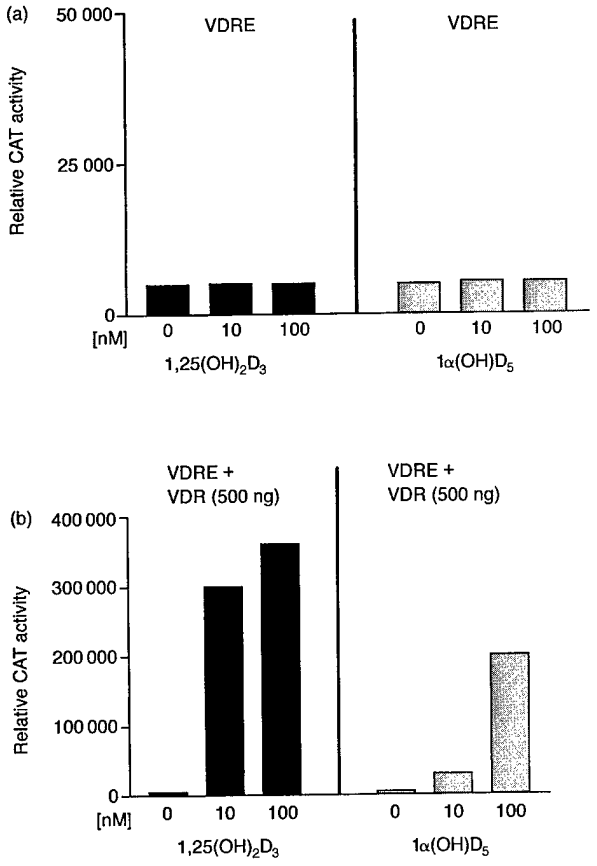

Fig. 4. Effects of $1,25(OH)D_3$ and $1\alpha(OH)D_5$ on the transactivation of the VDRE-conjugated reporter gene. Transient transfections of CV-1 cells with either VDRE-tk-CAT alone (a) or with VDR (b) was carried out by the calcium phosphate precipitation procedure. The cells were incubated with 10 and 100 nM vitamin D analogues for 3 days. CAT activity was measured spectrophotometrically. The experiments were repeated twice.

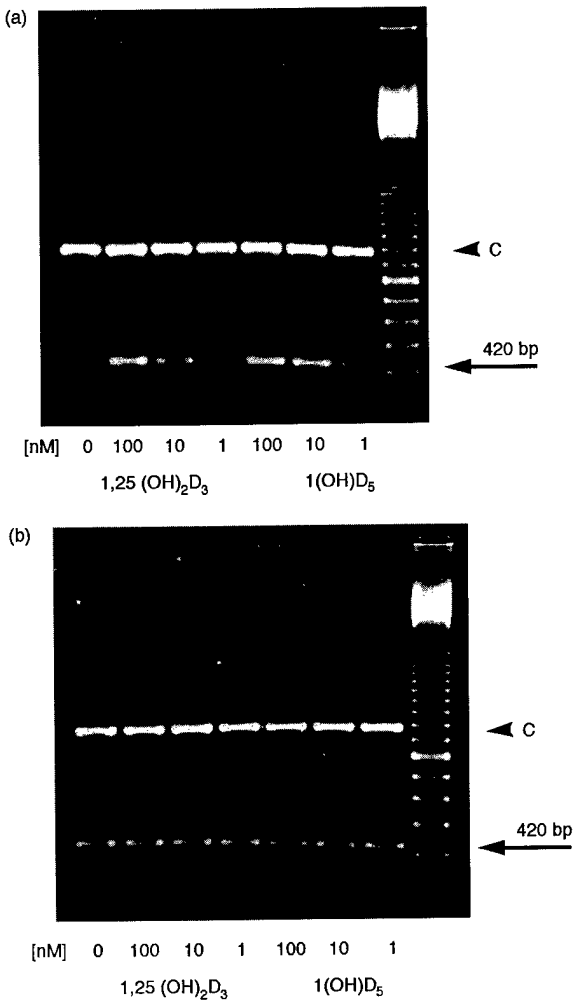

Fig. 5. Effects of vitamin D analogues on the expression of VDR mRNA in $MCF10_{neo}$ and T47D cells. Cells were incubated with various concentrations of analogues for 3 days in culture as previously described. VDR expression was measured by RT-PCR in (a) T47D cells; (b) $MCF10_{neo}$ cells. C, control housekeeping gene.

levels of VDR, do not respond to active vitamin D analogue(s) [24]. The VDR-mediated transcription regulatory genes include *TGFβ*, *EGF*, *c-myc* [25,26], and cell cycle regulators. The effects of various vitamin D analogues on programmed cell death have been evaluated in a variety of breast cancer cell lines. Consistently, MCF-7 cells which are ER+, VDR+ and positive for wild-type p53 exhibit apoptosis in response to vitamin D [27,28]. Although considerable literature exists for vitamin D-induced differentiation, its clinical application has been limited. This is due to its cytotoxicity at the concentration that induces differentiation. To this end, we have identified an analogue of the vitamin D_5 series which is non-calcaemic at the concentration at which 1,25-dihydroxyvitamin D_3 would induce hypercalcaemia. We previously reported that 1α-hydroxyvitamin D_5 inhibits carcinogen-induced development of mammary lesions in culture [12]. We also reported that it induces VDR and TGFβ in mammary epithelial cells. In this report, we addressed the question, "Does 1-hydroxyvitamin D_5 induce cell differentiation of breast cancer cells to the same extent as the active metabolite of vitamin D, 1,25-dihydroxyvitamin D_3?". T47D and $MCF10_{neo}$ cells were selected for the present study, since T47D cells are ER- and progesterone (PR)-positive and $MCF10_{neo}$ cells are negative for both ER and PR. Both analogues of vitamin D, $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$, inhibited cell proliferation to the same extent and induced differentiation as determined by the increased expression of differentiation markers.

The $MCF10_{neo}$ cells were originally derived from normal breast tissue and the epithelial cells were subsequently immortalised. The $MCF10_{neo}$ cells are ER-VDR+ and stably transfected with ras. The cells are tumorigenic in athymic mice. Since both T47D and $MCF10_{neo}$ have similar VDR and p53 status and differ only in their ER status, we compared the response of T47D ER+ and $MCF10_{neo}$ cells to two analogues of vitamin D. The $MCF10_{neo}$ cells, like T47D cells, exhibited a suppression of cell proliferation; however, no induction of differentiation was noticed. This, therefore, raised the question of whether induction of VDR is essential for cell differentiation. We evaluated the induction of VDR mRNA by these two vitamin D analogues. The results showed that $MCF10_{neo}$ cells constitutively expressed VDR-mRNA. However, there was no induction of the VDR message by either of the vitamin D analogues. In contrast, there was a dose-dependent increase in the expression of VDR mRNA in the T47D cells by both vitamin D_3 and D_5 analogues. These results suggest that there may be a positive association between the differentiation of cells by vitamin D and the induction of vitamin D-induced mRNA of VDR. Alternatively, the antiproliferative effects may be mediated by p53 although this is most unlikely in this case as both $MCF10_{neo}$ and T47D cells do not have functional p53 [10] and yet they respond to antiproliferative activity of vitamin D analogues. These results suggest that the antiproliferative effects and differentiating effects of vitamin D analogues may be independent of the cellular p53 status. These results are consistent with a recent report indicating the non-involvement of p53 in vitamin D-mediated differentiating/cell growth suppressing functions in breast cancer cells. Thus, it is not clear what mechanism may be operative for the suppression of cell growth by vitamin D analogues. If both antiproliferative effects and cell differentiating effects are mediated by VDR, then it is possible that the constitutive level of VDR will be sufficient to mediate vitamin D's effects in suppressing cell proliferation but that induction of new VDR mRNA may be necessary for cell differentiation.

Comparison between the action of a natural ligand of vitamin D, $1,25(OH_2)D_3$, and a vitamin D_5 analogue was also made in terms of their ability to transactivate a VDRE-reporter *CAT* gene. We selected VDR-negative CV-1 cells for these studies so that the endogenous VDR would not interfere with the interpretation of data. Since CV-1 cells are truly VDR-negative, they do not respond to incubation with $1,25(OH_2)D_3$ and do not transactivate the VDRE-CAT reporter. Since both T47D and $MCF10_{neo}$ cells express basal levels, to different extents, of VDR, the vitamin D analogue-induced transactivation of the CAT reporter may vary between these two cells and will compromise comparing the two analogues of vitamin D. Results showed that both $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ bind VDR and interact with VDRE. It was noted that, with 500 ng VDR transfection into CV-1 cells, $1,25(OH)D_3$ at 10 nM induced the reporter expression by more than 150-fold compared with the induction by $1\alpha(OH)D_5$ at the same concentration. The results indicate that, at equimolar concentrations, $1,25(OH)_2D_3$ is more potent in transactivating the *VDRE* reporter gene than $1(OH)D_5$. This is consistent with the earlier findings that, in mouse mammary gland organ cultures, the D_5 analogue is required at a log molar higher concentration to achieve similar effects to those observed with $1,25(OH)_2D_3$. The advantage, however, is that the D_5 analogue does not induce unwarranted toxicity which is often associated with 1,25-dihydroxyvitamin D_3. These studies collectively indicate that the vitamin D_5 series of agents mediate their action via the same VDR-mediated mechanism that is operative with the active metabolite of vitamin D_3.

Acknowledgements

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References

1. Colston KW, Chander SK, Mackay AG, Coombes RC. Effect of synthetic vitamin D analogues on breast cancer cell proliferation *in vivo* and *in vitro*. *Biochem Pharmacol* 1992, **44**, 693–702.
2. Danielpour D, Kadomatsu K, Anzano MA, Smith JM, Sporn MB. Development and characterization of nontumorigenic and tumorigenic epithelial cell lines from rat dorsal lateral prostate. *Cancer Res* 1994, **54**, 3413–3421.
3. Colston KW, Berger U, Coombes RC. Possible role for vitamin D in controlling breast cancer cells proliferation. *Lancet* 1989, **28**, 188–191.
4. Bower M, Colston KW, Stein RC, *et al.* Topical calcipotriol treatment in advanced breast cancer. *Lancet* 1991, **337**, 701–702.
5. Eisman JA, Barkla DH, Tutton PJM. Suppression of *in vivo* growth of human cancer solid tumor xenografts by $1,25(OH)_2D_3$. *Cancer Res* 1987, **47**, 21–25.
6. Sato T, Tagusagawa K, Asoo N, Konno K. Effects of 1-α-hydroxyvitamin D_3 on metastasis of rat ascites hepatoma K-231. *Br J Cancer* 1984, **50**, 123–125.
7. Elstner E, Linker-Israeli M, Said J, *et al.* 20-epi-vitamin D_3 analogues: a novel class of potent inhibitors of proliferation and inducers of differentiation of human breast cancer cell lines. *Cancer Res* 1995, **55**, 2822–2830.
8. Norman AW, Zhou JH, Henry HL, Uskokovic MR, Koeffler H. Structure–function studies on analogues of 1 a,25-dihydroxy-vitamin D_3: differential effects on leukemic cell growth, differentiation, and intestinal absorption. *Cancer Res* 1990, **50**, 6857–6864.
9. Frappart L, Falette N, Lefebvre M, Bremond A, Vauzelle JL, Saez S. *In vitro* study of effects of 1,25-dihydroxyvitamin D_3 on the morphology of human breast cancer cell line BT 20. *Differentiation* 1989, **40**, 63–69.
10. Mathiasen IS, Colston KW, Binderup L. EB 1089, a novel vitamin D analogue, has strong antiproliferative and differentiation inducing effects on cancer cells. 1. *J Steroid Biochem Mol Biol* 1993, **46**, 365–371.
11. Napoli JL, Fiivzzani MA, Schnoes HK, DeLuca HF. Synthesis of vitamin D_5: its biological activity relative to vitamins D_3 and D. *Arch Biochem Biophys* 1979, **197**, 119–125.
12. Mehta RG, Moriarty RM, Mehta RR, *et al.* Prevention of preneoplastic mammary lesion development by a novel vitamin D analogue, 1a-Hydroxivitamin D_5. *J Natl Cancer Inst* 1997, **89**, 212–218.
13. Mithasen IS, Laderman U, Jattela M. Apoptosis induced by vitamin D compounds in breast cancer cells is inhibited by bcl-2 but does not involve capases or p53. *Cancer Res* 1999, **59**, 4848–4856.
14. Haussler MR. Vitamin D receptors: nature and function. *Ann Rev Nutr* 1986, **6**, 527–562.
15. Yu VC, Delsert C, Andersen B, *et al.* RXRβ: a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. *Cell* 1991, **67**, 1251–1266.
16. Kliewer SA, Umesono K, Mangeldorf DJ, Evans R. Retinoid X receptors interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D_3 signaling. *Nature* 1992, **355**, 446–449.
17. Ross TK, Moss VE, Prahl JM, DeLuca HF. A nuclear protein essential for binding of rat 1,25-dihydroxyvitamin D_3 receptor to its response elements. *Proc Natl Acad Sci USA* 1992, **89**, 256–260.
18. Umesono K, Murakami KK, Thompson CC, Evans R. Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D_3 receptors. *Cell* 1991, **65**, 1255–1266.
19. Ross TK, Darwish HM, Moss VE, DeLuca HF. Vitamin D-influenced gene expression via a ligand-independent, receptor-DNA complex intermediate. *Proc Natl Acad Sci USA* 1993, **90**, 9257–9260.
20. Cheskis B, Freedman LP. Ligands modulate the conversion of DNA-bound vitamin D_3 receptor (VDR) homodimers into VDR-Retinoid X receptor heterodimers. *Mol Cell Biol* 1994, **14**, 3329–3338.
21. Agadir A, Lazzaro G, Zheng Y, Zhang X-K, Mehta RG. Resistance of HBL100 human breast epihthelial cells to vitamin D action. *Carcinogensis* 1999, **20**, 577–582.
22. Hecker KH, Roux KH. Touch down RT-PCR for better resolution in BioTechniques 1996, **20**, 478–485.
23. Wu Q, Li Y, Liu R, Agadir A, Lee -M, Liu Y, Zhang X-K. Modulation of retinoic acid sensitivity in lung cancer cells by a dynamic balance nur77 and COUP-TF orphan receptors and their heterodimerization. *EMBO J* 1997, **16**, 1656–1669.
24. Buras RR, Schmaker LM, Brenner RV, Shabahang M, Nauta RJ, Evans SR. Vitamin d receptors in breast cancer cells. *Breast Cancer Res Treat* 1994, **31**, 191–202.
25. Darwish H, DeLuca HF. Recent advances in the molecular biology of vitamin action. *Prog Nucl Acid Res Mol Biol* 1996, **53**, 321–344.
26. Simpson RU, Hsu T, Wendt MD, Taylor JM. 1,25-Dihydroxy-vitamin D_3 regulation of c-myc protooncogene transcription. *J Biol Chem* 1989, **264**, 19710–19715.
27. Welsh J, VanWeelden K, Flanagan L, Nolan E, Narvaez CJ. The role of vitamin D_3 and antiestrogens in modulating apoptosis of breast cancer cells and tumors. *Subcell Biochem* 1998, **30**, 245–270.
28. Simboli-Campbell M, Navaez CJ, van Weelden K, Tenniswood M, Welsh J. Comparative effects of $1,25(OH)_2D_3$ and EB1089 on cell cycle kinetics and apoptosis in MCF-7 breast cancer cells. *Breast Cancer Res Treat* 1997, **42**, 31–41.